Clinical, Pathological, Biochemical and Genomic Characteristics of Poorly Differentiated Thyroid Cancer

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Research presented in this thesis has been performed on the Head and Neck Service of the Department of Surgery (Head: Prof.dr.J.P.Shah), the Endocrinology Service Research Laboratory of the Department of Medicine and the Human Oncology and Pathogenesis Program (Head: Prof.dr.J.A.Fagin), and the Department of Pathology, Head and Neck pathology section (Head: Dr.R.A.Ghossein), Memorial Sloan Kettering Cancer Center, New York, USA

Cover: The Dresden Mayan Codex (Codex Dresdensis), the oldest surviving book of the Americas dating to the 13th-14th century, containing complex mathematical and astronomical calculations of exceptional accuracy.

Layout by Nauka

ISBN: 978-94-91688-10-2

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op dinsdag 11 december 2018, te 10.00 uur

door

Tihana Ibrahimpašić

geboren te Zagreb, Republiek Kroatië

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Chapter 1

Introduction

Chapter 1: INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and the fifth most common cancer in women with estimated annual incidence of 56 870 cases in the USA (1). Two biologically distinct classes of thyroid cancer exist based on their origin from follicular or parafollicular C-cells. Follicular thyroid cells give rise to differentiated, poorly differentiated and anaplastic thyroid cancer, while parafollicular C-cells give rise to medullary cancer.

Cancers of follicular cell origin are the most prevalent type of thyroid cancer. Specifically, differentiated thyroid cancer accounts for more than 90% of thyroid cancer cases (2). However, poorly differentiated and anaplastic cancers are relatively rare, accounting for 1-15% (3,4) and 2-3 % of cases respectively (2) (***Figure 1***). Poorly differentiated thyroid cancer (PDTC) arises de novo or develops from differentiated thyroid cancer (DTC) and can further progress into anaplastic thyroid cancer (ATC). PDTC therefore occupies an intermediate position on a progression spectrum from DTC to ATC both in terms of cellular differentiation and clinical behavior. PDTC is biologically more aggressive compared to DTC but not as invariably lethal as ATC. Since DTC generally carries an excellent prognosis, PDTC is the major cause for dismal outcome in non-anaplastic thyroid carcinomas. Despite its clinical significance, studies on PDTC have been limited. One reason is the relative rarity of PDTC which mandates reports from high volume tertiary care institutions. In addition, diagnostic criteria on PDTC have been surrounded by controversy ever since the recognition of PDTC as a separate entity. Due to the high clinical significance of PDTC among non-anaplastic thyroid cancers it is important to improve our understanding of the specific biology that underlines PDTC clinical behavior. By having access to a high volume of thyroid cases in a tertiary care academic institution that has a long tradition of thyroid cancer treatment and research, we decided to study PDTC in detail with special emphasis on their clinical, biochemical, clinicopathologic and genomic characteristics.

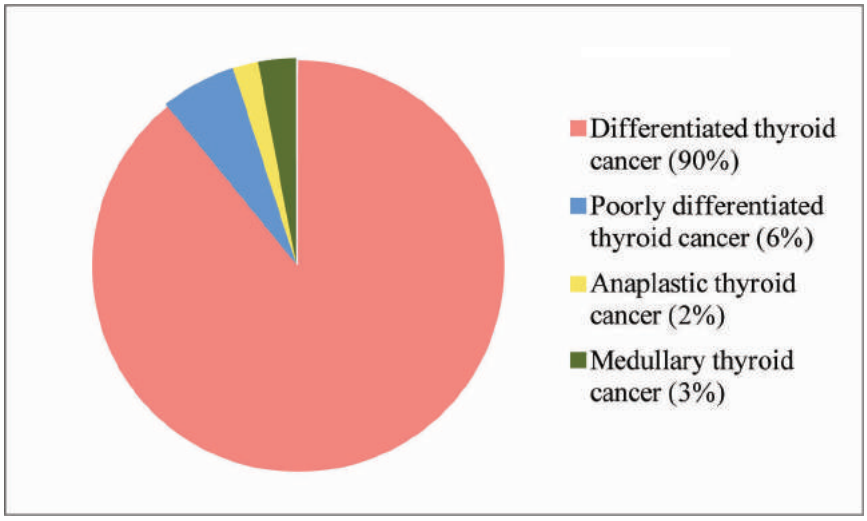

Figure 1

Defining Poorly Differentiated Thyroid Cancer

In 1907, Langhans described a type of malignant epithelial thyroid tumor with strikingly nesting pattern and named it "wuchernde struma" (proliferative goiter) *(**Figure 2**)*(5). In 1963, Granner et al. introduced the term poorly differentiated carcinoma of the thyroid (6). However, over the next few decades many poorly differentiated cancers were categorized as conventional follicular carcinoma due to their ability to form follicles (7). In this regard the Armed Forces Institute of Pathology (AFIP) stated that follicular carcinomas could be subdivided based on the degree of follicle formation and that diminished follicle formation correlated with aggressiveness and invasion (8).

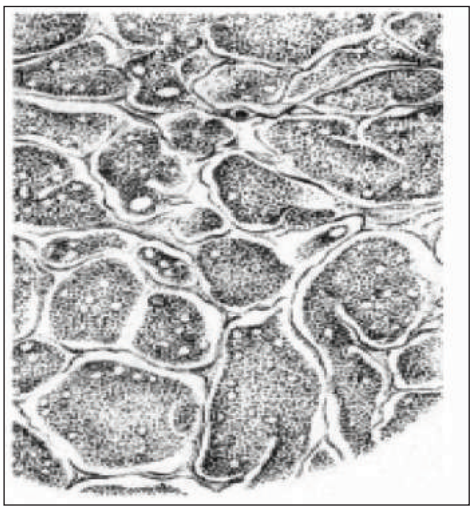

Figure 2 | Langhans "Wuchernde Struma" (1907)*: nesting pattern with small follicular lumina and cribriform configuration.

**reprinted by permission from Springer Nature, Virchows Arch (Pathol Anat), Über die epithelialen Formen der malignen Struma, Theodor Langhans, 1907*

In 1983, Sakamoto et al. characterized solid, trabecular and/or scirrhous pattern of growth without the presence of an anaplastic component as poorly differentiated thyroid cancer, which carried a worse prognosis compared to DTC (9). Carcangiu et al. revisited the term "wuchernde struma" in 1984 (10) and concluded that they are likely dealing with the same entity as Langhans. Carcangiu described her series as poorly differentiated thyroid cancer and adopted the term "insular pattern" based on similarities to nests formation ("insulae") in carcinoid tumors. Furthermore, Carcangiu also emphasized consistent presence of mitotic activity, frequent necrotic foci and capsular and blood vessel invasion in such tumors (10). Both Sakamoto and Carcangiu suggested that poorly differentiated thyroid cancer represents a separate clinicopathological entity that falls morphologically and in terms of survival between differentiated and anaplastic cancer. However, PDTC was not officially recognized as a separate entity until twenty years later. In 2004, WHO Classification of Endocrine Tumors (11) introduced PDTC as a diagnostic entity and described it as a follicular cell neoplasm, with limited evidence of follicular cell differentiation and with intermediate position both morphologically and in terms of behavior between differentiated (follicular and papillary carcinomas) and anaplastic cancer (11). Nevertheless clear criteria on the histopathological diagnosis of PDTC were missing and many reports on PDTC also included variants of DTC (e.g. columnar, Tall cell, oncocytic and diffuse slerosing variants) (4). Furthermore, a number of PDTC were also misinterpreted as medullary carcinoma (12) or ATC. Compared to PDTC, medullary carcinoma has negative thyroglobulin stains and irregular configuration of nest architecture,

together with more abundant and granular cytoplasm, positive amyloid, argyrophil granules and calcitonin (7). ATC compared to PDTC often shows pleomorphic giant and spindle cell patterns.

In 2006, the Turin consensus proposed diagnostic criteria for PDTC for the first time (13). These included: 1) solid/trabecular/insular pattern of growth, 2) absence of conventional nuclear features of papillary carcinoma and 3) at least one of the following features: convoluted nuclei, mitotic activity ≥3/10 HPF and tumor necrosis. The Turin proposal therefore considers both architecture and high-grade features as criteria for PDTC diagnosis. However solid, trabecular and insular architecture may also have distinctive geographical distribution. If PDTC is diagnosed based on solid, trabecular and insular patterns of growth, its prevalence is relatively rare in North America (2-3% of thyroid cancer cases) compared to a relatively high prevalence in Northern Italy (up to 15% of thyroid cancer cases) (4). Therefore, the diagnosis of PDTC based on growth pattern may underestimate this type of thyroid cancer in certain geographical areas. Furthermore, the Turin criteria may allow for biologically heterogenous categorization of PDTC which would imply that not all PDTC occupy an intermediate position between DTC and ATC. Pattern of growth may be debatable if we consider reports from papillary thyroid carcinoma (PTC). Solid growth may represent loss of more differentiatied pattern of growth, such as papillary and follicular growth. However, solid pattern of growth in PTC was reported in 34% of children post Chernobyl nuclear accident (14,15), in 15% of young children in UK (16) and also in 2% of adult patients with PTC (17). However, no excess mortality was found in children with the solid subtype (14). In adults, solid variant of PTC was associated with slightly higher frequency of distant metastases and less favorable prognosis than classical PTC (17), nevertheless solid variant of PTC did not reflect lower survival rates of PDTC. Nikiforov et al. suggested that PDTC and solid variant of PTC should be distinguished as separate entities (17). Solid pattern of growth (defined by Nikiforov et al. as solid/trabecular/insular pattern (17)) may therefore not be representative of tumor aggressiveness in PDTC based on studies on solid variant of papillary thyroid carcinoma (PTC).

On the other hand, histological grade was shown to categorize a more homogenous subsets of thyroid carcinomas. In PTC, histologic grade was a strong and independent prognostic marker while morphological subtypes had only minor prognostic impact (18). In PDTC defined by solid/trabecular/insular pattern, Volante et al. found that besides age, presence of necrosis and mitotic count >3/HPF were significantly associated with poor outcome (19). The most aggressive subgroup of PDTC in Volante's study (based on solid/trabecular/insular pattern and subclassified by grading parameters) (19) had comparable survival rates to PDTC diagnosed on the basis of mitosis and necrosis reported by Hiltzik et al (20). Therefore, definition of PDTC on the basis of grade, i.e. necrosis and high mitotic rate, represents a group of tumors that are more aggressive and more homogeneous than PDTC defined by growth pattern (20). According to Memorial Sloan Kettering Cancer Center (MSKCC) criteria, PDTC is defined as thyroid carcinoma with follicular cell differentiation (on routine microscopy and/or by immunohistochemisty, e.g. thyroglobulin positivity), presence of ≥5 mitosis/10 high-power microscopic fields (HPF) (X400) and/or fresh tumor necrosis (20) ***(Figure 3)***.

In our study on PDTC we employed the MSKCC diagnostic criteria based on proliferative grading for the following reasons: 1) PDTC defined on the basis of mitosis and necrosis constitutes biologically more homogenous group of tumors. This is reflected in the fact that the most common cause of radioiodine refractory fluorodeoxyglucose-PET-positive thyroid cancer is PDTC, defined on the basis of mitosis and necrosis (21) and the same relationship has not been shown for other definitions of PDTC, 2) diagnosis of PDTC by proliferative grading has the ability to predict intermediate prognosis as validated by the study of Gnemmi et al. (22) and 3) diagnosis of PDTC by proliferative grading is a simple and reliable method.

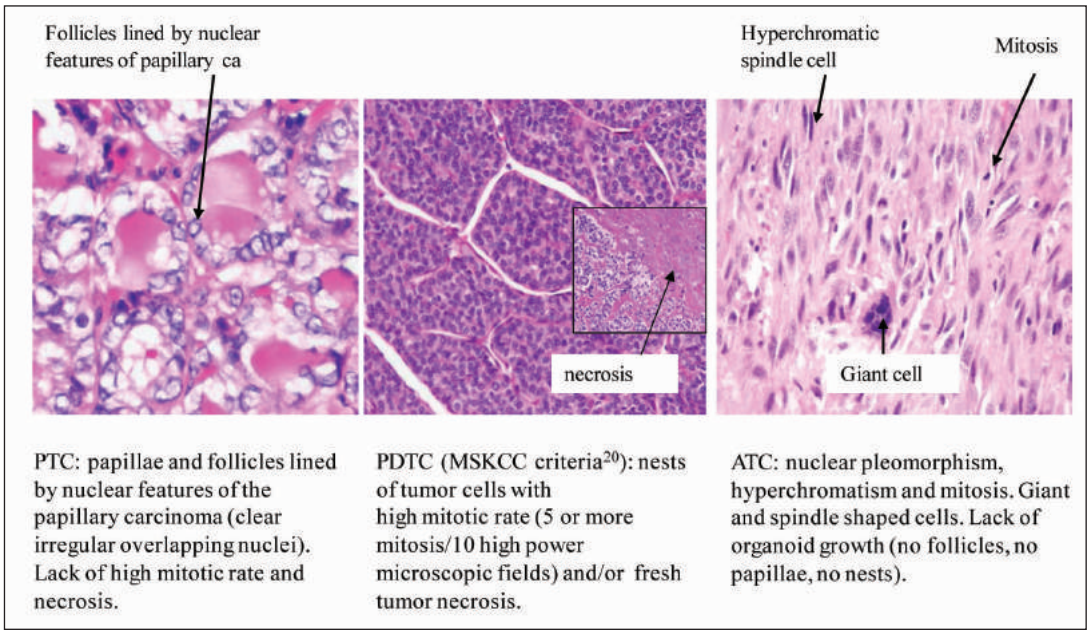

Figure 3 | Histopathologic progression of follicular cell-derived thyroid cancer
(courtesy of Dr. Ghossein, MSKCC)

Intermediate position of Poorly Differentiated Thyroid Cancer in the Progression Spectrum of Thyroid Cancers: Clinical Aspects

Thyroid tumors of follicular cell origin display various degrees of differentiation which in general correlates with their clinical aggressiveness. The progression of thyroid cancer from DTC to ATC has been well established. Relatively often in the case of PDTC or ATC, well differentiated morphology is found admixed with poorly differentiated or undifferentiated areas in the same tumor (23) or in the case of previously treated differentiated tumor, recurrence presents as poorly differentiated or undifferentiated tumor (23-25). Already in the early 1980s Sakamoto (9) and Carcangiu (10) suggested an intermediate position of PDTC between DTC and ATC, both in terms of morphology and clinical behavior. Due to the relative rarity of PDTC, prospective studies on the clinical behavior of PDTC are impractical and not feasible. There is level IV evidence that the prognosis of PDTC is intermediate between DTC and ATC (4), based on retrospective or cohort studies according to the classification of Sackett et al. (26). In general, 5 year thyroid cancer survival rates exceed 98% (27). This is attributed to the fact that the most common form of thyroid cancer is DTC, particularly the subtype papillary thyroid carcinoma (PTC), which has excellent prognosis in more than 80% of cases with a 20 year cause-specific mortality rate of <1% (28). On the other side of the spectrum is ATC, which is fortunately rare, comprising only 2% of all thyroid cancers, but with a fulminant course and average survival of 6 months (29). PDTC has been categorized as an intermediate clinical entity between DTC and ATC.

When compared to DTC, PDTC often presents at an older age and at an advanced stage (30). PDTC has a more aggressive course compared to DTC regardless of focal or diffuse presence of PDTC (19), with a higher propensity for local recurrence (30). As tumors progress from differentiated, to poorly differentiated and eventually to anaplastic cancer, significant progression is also observed in: tumor size, extrathyroidal extension (ETE), lymph node metastasis and distant metastasis (30,31). In the study on DTC and PDTC, age and

ETE were independently correlated with the presence of PDTC (30). However, clinical risk factors for outcome in PDTC have not been as elaborately defined as they are for DTC. Several prognostic scoring systems have been developed for differentiated thyroid cancer of follicular cell origin in order to improve survival prediction such as EORTC (32), GAMES (33), AMES (34), AGES (35), and MACIS (36). They all include age, ETE and distant metastatic involvement. The majority include tumor size and histologic type and some include gender, nodal metastasis and histologic grade as prognostic factors for survival. Due to the small number of PDTC patients and the heterogenous criteria for PDTC diagnosis, similar prognostic systems have not been developed for PDTC. However, it is generally believed that the same risk factors influence the prognosis of PDTC as those that are relevant for DTC. Similar to DTC, age was reported as a significant clinical prognostic factor in PDTC (37) In addition, tumor size larger than 4 cm and extracapsular extension were both associated with higher risk of distant metastases and poor survival in PDTC (20). Grade with regards to necrosis and mitotic count was also associated with poorer outcome in PDTC (19). 5 year disease free survival (DFS), disease specific survival (DSS) and overall survival (OS) were found to significantly differ between these three entities: they were best for DTC, intermediate for PDTC and worst for ATC (31). This pattern was confirmed in additional studies where statistically significant reduction in survival was found between DTC and PDTC (38) or between DTC, PDTC and ATC (9). However, studies that encompass larger cohorts of PDTC patients from tertiary care institutions are necessary to elucidate clinical characteristics of PDTC in more detail and to evaluate impact of clinical characteristics on the outcome. This was one of the aims of this thesis.

Today, treatment of DTC has been standardized. However, treatment of PDTC has not been standardized and therapeutic decisions on PDTC have mainly been extrapolated from treatment experience on DTC. When compared to DTC, PDTC does not usually respond well to the same treatment strategies Surgery is the treatment of choice for PDTC, but the extent of surgery for the primary tumor and regional lymph nodes in the neck has not been well established. Furthermore, criteria for postoperative therapy and its effectiveness have not been clear. Radioiodine (RAI) avidity of PDTC is variable (20) and while some studies report RAI uptake by PDTC (39), others did not find significant impact of RAI on survival in PDTC (40). Older age and extrathyroid extension and/or extranodal extension are associated with aggressive biology and loss of RAI avidity in differentiated thyroid cancer (41). PDTC often presents at an older age and at an advanced stage (30) which are both manifestations of more aggressive biology and thus can be accompanied by loss of RAI avidity in PDTC. The role of postoperative external beam radiation therapy (EBRT) in PDTC is equally controversial. EBRT can be beneficial in patients with DTC that have high risk of locoregional recurrence (42). Similar criteria could be applied to PDTC whereby EBRT has been recommended in PDTC patients with T3 tumors without distant metastasis, with T4 tumors and in all patients with neck node involvement (4). However, no significant survival improvement has been recorded in PDTC patients following EBRT (4,43). Regarding chemotherapy in PDTC, reports have been scarce. There is level III evidence (nonrandomized trials with contemporaneous controls according to Sackett et al. (26)) with short follow- up, that patients with inoperable PDTC who received chemotherapy regimen with or without EBRT became operable or free of disease (44). However, lack of studies on PDTC and small patient numbers as well as heterogenous diagnostic criteria prevent generalization on the efficacy of PDTC treatment. Due to the lack of systematic studies on PDTC the exact cause of death (locoregional failure or distant metastases) in PDTC is unclear. The cause of death could point to where the therapeutic efforts should be concentrated in order to obtain improved disease control and outcome. This was another aim of this thesis.

Postoperative follow-up strategies for PDTC are also not well established as they are for DTC. Thyroglobulin (Tg) is a glycoprotein that is normally produced by thyroid follicular cell. It acts as a scaffold for combining tyrosine residues with iodine in order to produce thyroid hormones and can be detected in the bloodstream as a byproduct of thyroid hormone synthesis (45). Normal thyroid tissue produces Tg. In addition, the majority

of DTC are also Tg positive even with metastatic cancer at presentation. Tg is therefore unique for thyroid tissue and also a sign of tumor differentiation. In contrast, ATC is mostly Tg negative or weakly positive on immunohistochemistry (46) due to loss of differentiation. PDTC still has some remnants of differentiation and some ability to produce Tg (47). However immunohistochemistry stainings of Tg are usually weak or focal in PDTC (13). This is due to tumor heterogeneity within the primary tumor. Weak Tg production supports the intermediate nature of PDTC between DTC and ATC. Tg has routinely been used as a postoperative follow-up tool in detecting the presence of residual disease or recurrence in DTC. However studies on Tg in PDTC have been limited (48,49) and whether undetectable Tg carries any correlation with the outcome in PDTC as it does in DTC has not been clear. This was another aim of this thesis.

Genomic profiling of PDTC in Light of Evolution of Sequencing Technologies: What We Know so Far on Molecular Characteristics of Thyroid Cancer

There has been an ongoing pursuit to elucidate molecular mechanisms behind the progression of thyroid carcinoma from DTC to ATC. Several driver genes and genetic pathways have been established mainly from studies on DTC, while reports on less differentiated thyroid cancers such as PDTC and ATC have been scarce and mostly limited due to small numbers of patients.

Gene aberrations and accompanying loss, gain or change of function in thyroid carcinomas have been found in many elements responsible for cell growth, differentiation and survival; e.g. cell surface receptors and signal transduction proteins, nuclear receptors, nuclear proteins, markers of follicular cell differentiation and function and molecules of focal adhesion and cell motility. Additionally, epigenetic mechanisms of modifying the gene function have also been reported. Driver mutations in thyroid cancer are mutually exclusive in well differentiated tumors and are often associated with particular histotype and specific clinicopathological characteristics.

CELL SURFACE RECEPTORS AND SIGNAL TRANSDUCTION PROTEINS

Thyroid proliferation and differentiation is mediated by several distinct signal transduction pathways. The most commonly studied have been tyrosine receptor kinase (TRK) pathways: TRK/RAS/RAF/MAPK (mitogen-activated protein kinase (MAPK) cascade) pathway is primarily involved in papillary thyroid carcinomas (PTC) and the PI3K/AKT/PTEN pathway is primarily involved in follicular thyroid carcinomas (FTC) (***Figure 4***). Both of these pathways promote proliferation and thyrocyte dedifferentiation.

MAPK signaling pathway

In thyroid cancer, constitutive activation of MAPK signaling pathway usually occurs due to point mutations in signaling proteins (*BRAF* or *RAS*) or as a result of tyrosine receptor kinase (TRK) rearrangements (involving *RET*, *NTRK1* or *ALK*). Different degrees of MAPK signaling induction has been observed depending on the type of activating mutation. BRAFV600E oncoprotein causes the greatest MAPK output because its monomeric nature escapes the negative feedback of downstream activated ERK (50). In contrast, TRK rearrangements and mutated RAS still receive negative feedback by ERK, which causes less intensive MAPK output than that induced by BRAFV600E (50). The most common genetic alterations in MAPK pathway are typically mutually exclusive in DTC, however simultaneous presence of BRAFV600E, RAS mutations and RET/PTC rearrangements have been reported in aggressive forms of thyroid cancer (recurrent PTC and ATC) (2).

BRAF mutations

RAF proto-oncogenes encode for intracellular serine-threonine protein kinases which are recruited to the plasma membrane by activated RAS leading to their phosphorylation and activation. Activated RAF kinase subsequently phosphorylates MEK which in turn phosphorylates ERK. Activated ERK translocates to the nucleus where it regulates transcription of genes involved in cell differentiation, proliferation and survival (***Figure 4***). BRAF is the predominant isoform in thyroid cells. 95% of all *BRAF* mutations in thyroid cancer represents T1799A transverse point mutation in exon 15 of the *BRAF* gene that causes V600E mutation of BRAF protein (51). Mutated amino acid 600 lies in the kinase domaine of BRAF and causes unrestrained BRAF kinase upregulation (51). Other rare types of BRAF mutations mostly affect nucleotides around codon 600 and also result in unrestrained BRAF kinase activation (52,53). In addition, various *BRAF* fusions with diverse gene partners have been described in PTC (54). While point mutations of *BRAF* dominate in sporadic PTC, radiation induced PTC are more likely to contain *BRAF* rearrangements, such as *AKAP9/BRAF* fusions (55). The latter results in enhanced kinase activity through conservation of the protein kinase domain while lacking the autoinhibitory N-terminal portion of BRAF.

BRAFV600E mutation is strongly associated with papillary thyroid cancer (52). Tall cell variant and classical papillary carcinoma tend to carry the highest rates of BRAF mutations (80% and 60% respectively), while the lowest rates are found in follicular variant of papillary thyroid cancer (FVPTC) (12%) (56). Encapsulated FVPTC with or without invasion is associated with RAS mutation, however infiltrative FVPTC tends to carry BRAF over RAS mutation (50). BRAF mutation has also been described in PDTC (15%) and ATC (up to 30%) (57). Presence of BRAF mutation in both well differentiated and poorly differentiated or anaplastic tumor areas, provides evidence that BRAF mutation represents an early event in tumorigenesis (57). Many individuals with mutated BRAF and PTC or papillary microcarcinoma go on to have an excellent prognosis. However, significant association between mutated BRAF and aggressive histopathological features in PTC has been shown and it has been difficult to determine whether BRAF mutation per se or aggressive histopathological features influence the risk of worse outcome (58). Furthermore studies have found a close association of mutated BRAF with dedifferentiation in PTC and decreased expression of thyroid-specific genes; e.g. sodium/iodide symporter (*NIS*), thyroperoxidase (*TPO*), pendrin (*SLC26A4*), thyroid-stimulating hormone receptor (*TSHR*) and Tg (*TG*) (59). These genes are generally silenced due to aberrant methylation promoted by mutated BRAF (60). Significantly lower expression of thyroid genes required for RAI incorporation (61) ultimately results in loss of RAI avidity in BRAF positive metastatic (62) or recurrent thyroid cancer (63). Nevertheless, the predictive value of mutated BRAF and its role in thyroid cancer progression is yet to be defined.

RAS mutations

RAS mutations are the second most common mutations in thyroid cancer after *BRAF* mutations. RAS oncoproteins normally transduce signals from the cell membrane receptors (TRK and G-protein-coupled receptors) to downstream targets in MAPK and PI3K/AKT signaling pathways. RAS has an intrinsic GTPase activity which acts as a molecular switch between RAS active state (GTP bound) and RAS inactive state (GDP bound) (64). In thyroid cancer, mutations of all three RAS isoforms have been reported (NRAS, HRAS and KRAS), however mutation of NRAS is the most common (65). RAS mutations typically occur at codon 12,13 or 61 (66); for KRAS typically at codon 12, NRAS at codon 61, while HRAS displays intermediate behavior (64). Some reports have suggested preferential association of mutated RAS with AKT phosphorylation in thyroid cancers (67,68), indicating preferential activation of PI3K/AKT signaling by mutated RAS in thyroid tumorigenesis.

RAS mutations are prevalent in follicular histotypes of thyroid neoplasia: in 20-40% of follicular adenomas (57), 25-57% of follicular thyroid carcinomas (FTC) (65,69,70) and in 25% of follicular variants of papillary thyroid

carcinoma (FVPTC) (71). RAS mutations are less prevalent in Hürthle cell carcinoma (11%) (72) and rare in classical variant of PTC. In less differentiated tumors RAS mutations have been reported in high percentages of PDTC (55%) and ATC (52%) (73) and are therefore present along the spectrum of thyroid tumors, from follicular adenomas to ATC (65). Mutated RAS may contribute to differentiation loss since it is found in DTC with areas of dedifferentiation and generally more frequently in PDTC and ATC than in DTC (74). In addition, an inverse correlation between RAS mutation and thyroglobulin expression has been reported (75). RAS mutations have been implicated in tumor initiation, as well as in tumor progression by modulating cell motility and invasiveness (76,77). The latter is reflected in a significant association between RAS mutations and presence of distant metastases (73). This correlates with the prevalence of RAS mutations in follicular patterned tumors such as FTC or FVPTC, which spread infrequently to regional lymph nodes and are prone to vascular invasion. Patients with RAS mutated tumors also tend to have significantly higher mortality rates and mutated RAS has been reported as an independent predictor of poor survival (73). Nevertheless the predictive value of RAS mutations has not been definitely established due to relative rarity of histotypes that tend to carry RAS mutations and its presence in both aggressive and relatively indolent types of thyroid cancer.

Tyrosine Receptor Kinase

***RET/PTC* rearrangements**

RET proto-oncogene encodes for receptor tyrosine kinase in cells of neural crest origin but is not expressed in normal thyrocytes. *RET/PTC* rearrangements occur when 3′ *RET* tyrosine kinase (TK) domain combines with the 5′ terminal region of genes that are constitutively expressed in thyroid follicular cells (78). Therefore this fusion initiates ligand-independent dimerization and constitutive activation of RET TK, recruitment of signaling adaptor proteins to phosphorylated tyrosine residues on intracellular domain of RET fusion protein (79) and downstream activation of MAPK and PI3K/AKT pathways (2). In thyroid cancer, the most common are *RET/PTC1* (partner gene of *RET* is coiled-coil domain- containing gene 6: *CCDC6,* also known as *H4)* and *RET/PTC3* (partner gene of *RET* is nuclear receptor co-activator 4: *NCOA4,* also known as *ELE1)* (2), which is explained by spatial contiguity of *RET* and the partner gene in the nucleus of thyrocytes (80).

RET/PTC is relatively frequent in papillary thyroid cancer and is also found in FVPTC and FTC. In FTC, *RET/PTC* is associated with oncocytic tumors that show solid growth pattern (81). In sporadic adult papillary thyroid cancer *RET/PTC* rearrangement is found in 10-20% of cases (57). However, it is more frequently detected in patients with a history of radiation exposure (50-80%) and in papillary cancer from children and young adults (40-70%) (82-84). *RET/PTC1* is associated with classical papillary cancer in children and young adults, which also displays slow proliferation and a more benign course (82-85). *RET/PTC3* is associated with solid variant of papillary cancer, frequently found in children and after radiation exposure and is more prone to display aggressive clinical features with regional and lung metastases (82-85). However despite the presence of advanced disease, solid variant of papillary cancer in young patients with or without *RET/PTC3* rearrangement shows a good response to RAI therapy and is not associated with significantly worse patient survival (86). In addition, prevalence of *RET/PTC* in less differentiated tumors like PDTC and ATC is low (86) and therefore *RET/PTC* may not have a significant role in thyroid cancer progression and dedifferentiation. Although the presence of *RET/PTC* was shown to correlate with high growth rate in benign thyroid tumors (87) the role of *RET/PTC* in thyroid tumorigenesis is yet to be determined.

***NTRK* rearrangements**

NTRK proto-oncogene encodes a member of the neurotrophic tyrosine kinase receptor (NTKR) family, which upon neurotrophin binding phosphorylates itself and members of the MAPK and PI3K/AKT pathway. *NTRK* is not expressed in normal thyrocyte and fusion of this proto-oncogene with a partner gene leads to

expression of tyrosine kinase oncoprotein in a thyroid cell. Somatic rearrangements of *NTRK1* gene in PTC are less common than those involving the *RET* gene: their frequency does not exceed 12% (88). However, *NTRK* gene rearrangements play a role in early events of thyroid carcinogenesis (89). Similar to *RET* rearrangements, *NTRK1* rearrangements have been associated with young age and advanced stage at presentation (90). *ETV6/NTRK3* was reported in FVPTC, in both encapsulated and infiltrative variants (91).

ALK mutations and rearrangements

Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor subfamily of TRK that can activate MAPK and PI3K/AKT pathways. Point mutations of ALK have been reported in 11% of ATC (92). Rearrangement of ALK with various partner genes such as *EML4* (echinoderm microtubule-associated protein-like 4) and *STRN* (Striatin) have been reported in PTC *(EML4/ALK* and *STRN/ALK)* and in PDTC and ATC *(STRN/ALK)* whereby both of these rearrangements result in dimerization and constitutive activation of ALK kinase (93).

MAPK related pathways in thyroid cancer

NF-κB pathway

In nuclear factor-κB (NF-κB) pathway extracellular stimuli from cell membrane receptors activate the inhibitor of κB (IκB) kinase (IKK) which phosphorylates IκB. Phosphorylated IκB dissociates from NF-κB, to which it is normally bound in the cytoplasm and undergoes degradation. NF-κB is free to enter the nucleus and act on tumour-promoting genes. BRAFV600E is thought to promote the phosphorylation of IκB and the release of NF-κB, thus activating the NF-κB pathway (2). In addition other members of MAPK pathway (like RAS and RET/PTC) can also cause activation of the NF-κB pathway in thyroid cancer (2).

RASSF1A-MST1-FOXO3 pathway

RASSF1–mammalian STE20-like protein kinase 1 (MST1)-forkhead box O3 (FOXO3) pathway is activated by extracellular pro-apoptotic stimuli through membrane receptors. Phosphorylated FOXO3 dissociates from 14-3-3 proteins. While 14-3-3 proteins undergo proteasomal degradation, phosphorylated FOXO3 enters the nucleus and promotes the expression of pro-apoptotic genes in the FOXO pathway. BRAFV600E directly inhibits MST1 and prevents its activation by RASSF1A, thereby suppressing the FOXO3 pathway and pro-apoptotic signaling (2).

HIF1α pathway

In response to hypoxia, HIF1α binds to HIF1β (also known as ARNT) to form the HIF1 transcription factor which induces genes responsible for cell metabolism and angiogenesis (94). HIF1α is not normally expressed in thyroid tissues but is expressed in aggressive types of thyroid cancer, whereby both the MAPK and the PI3K/AKT pathways can upregulate HIF1 (2).

PI3K/AKT/PTEN pathway

When the ligand binds to the tyrosine kinase receptor, RAS is activated and recruits PI3K to the cell membrane by binding its catalytic subunit (e.g. PIK3CA). The catalytic subunit of PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) and PIP3 activates 3-phosphoinositide-dependent kinase 1 (PDK1) which in turn phosphorylates and activates AKT. Phosphorylated AKT enters the nucleus and induces tumor-promoting genes. In addition phosphorylated AKT plays a role in activating other signaling pathways in the cytoplasm, such as the mammalian target of rapamycin (mTOR) pathway, which promotes translation (2).

AKT-1 and AKT-2 are prevalent isoforms of this serine-threonine kinase in thyroid cancer (95) with a particular role of AKT-1 in FTC (96). Tumor supressor *PTEN* acts as the major negative regulator of the PI3K/AKT pathway, acting through PIP3 dephosphorylation (97) (***Figure 4***). Mutation or deletion of tumor suppressor *PTEN* results in constitutive activation of PI3K/AKT pathway and development of thyroid cancer (particularly FTC). Furthermore methylation and thus silencing of *PTEN* can be caused by aberrant activation of PI3K/AKT signaling pathway itself, in effect creating a self-amplifying loop (2). Thyroid cancer (particularly FTC, PDTC, ATC) can also result from mutations in *PIK3CA* (which encodes p110α catalytic subunit of PI3K and represents the isoform implicated in human cancers) (2,98). In addition, mutations *of AKT1* have been reported in metastatic thyroid cancer (62). Similarly to MAPK pathway activation by BRAFV600E, PI3K/AKT pathway activation can also suppress iodide incorporation in thyroid cells (99). This is in concordance with induction of the expression of genes responsible for iodide incorporation in thyroid cancer cells after inhibition of PI3K/AKT pathway (100).

While overactivation of MAPK signaling dominates in the development and progression of PTC, overactivation of PI3K/AKT signaling dominates in the development and subsequent progression of FTC (2).

PI3K/AKT related pathways in thyroid cancer

RASSF1A-MST1-FOXO3 pathway

In FTC, this pro-apoptotic pathway is downregulated through AKT-mediated phosphorylatation of forkhead box O3 (FOXO3) whereby FOXO3 is translocated from the nucleus and sequestered in the cytoplasm by 14-3-3 proteins (2).

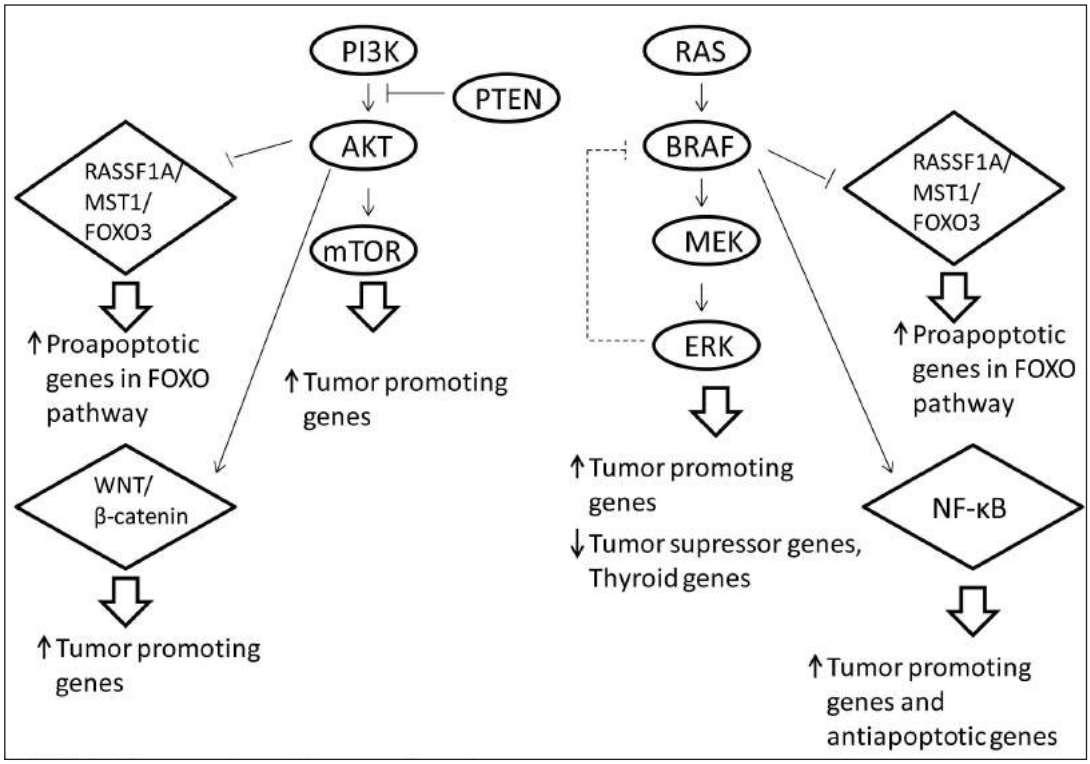

Figure 4 | Main signaling pathways in thyroid cancer

WNT–β-catenin signalling pathway

Phosphorylated AKT further phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β). This lifts GSK3β-mediated suppression of β-catenin and β-catenin can enter the nucleus to induce tumour-promoting genes. WNT–β-catenin signaling can also be activated by RET/PTC, either through direct phosphorylation of β-catenin or through activation of PI3K/AKT pathway in thyroid cancer (101). WNT–β-catenin pathway may have an important role in thyroid cancer progression since mutations of *CTNNB1* (encodes for β-catenin) have been reported in up to 20% PDTC and 70% ATC (57), whereby higher expression of β-catenin was reported in ATC compared to DTC (2).

HIF1α pathway

In a response to tumor hypoxia, PI3K/AKT pathway can upregulate HIF1 as in the case of MAPK pathway (2).

NUCLEAR RECEPTORS

***PAX8/PPARγ* rearrangements**

The *PAX8/PPARγ* fusion results in a high expression of chimeric PAX8/PPARγ protein (PPFP) which transactivates transcription factor PAX8 – responsive genes and inhibits PPARγ tumor suppressor activity in a dominant-negative effect (102). 30-35% of FTC and less than 5% of FVPTC carry *PAX8/PPARγ* rearrangement (57). Less than 3% of FTC show a different type of rearrangement, *CREB3L2/PPARγ,* with deregulation of CREB3L2 transcription signaling (103).

MARKERS OF FOLLICULAR CELL DIFFERENTIATION AND THYROCYTE FUNCTION

Immunohistochemical positivity for **thyroglobulin (Tg)** and **thyroid transcription factor 1** (**TTF-1**) has been detected in most PDTC, though this is generally weak (9). Expression of functional molecules such as **thyroperoxidase** (**TPO**) was found to be absent or decreased on immunohistochemistry in both differentiated and undifferentiated tumors (104-106). Immunohistochemistry also revealed decreased **TSH receptor** expression in PDTC, in correlation with high proliferation as measured by MIB-1 (107) .

NUCLEAR PROTEINS THAT CONTROL THE CELL CYCLE

p16 and cyclins

p16 is cyclin dependent kinase inhibitor that acts as tumor suppressor. Higher aberrant promoter methylation of gene encoding for p16 *(CDKN2A)* was found in PTC compared to normal and benign tissue (108), while p16 expression varied in PDTC (109). Cyclins activate cyclin dependent kinase and cyclins A, B1, and E were expressed on immunohistochemistry in more than 70% of PDTC (110), while cyclin D1 was found overexpressed in both PTC and FTC (111) and in PDTC and AC (112).

p53

TP53 encodes tumor suppressor protein p53 which has an important role in cell cycle control, apoptosis and DNA repair. p53 is a transcription factor for p21 which binds cyclin-CDK complexes thus inhibiting their kinase activity and arresting cell division. This stop signal allows for DNA repair or apoptosis (113). *TP53* mutations usually affect exons 5-9 of chromosome 17 (typically affecting codon 273), resulting in dominant-negative mutant p53 protein or less frequently in total p53 absence (86). *TP53* mutations and /or mutant p53 expression have rarely been encountered in DTC, whereby they are mostly associated with aggressive variants of PTC (tall cell, mixed columnar, micropapillary/hobnail or cribriform-morular variant of PTC) (86). However, up to 30% of

PDTC and 80% of ATC showed *TP53* mutations (57), implying association of mutated p53 with dedifferentiation and more aggressive behavior.

MOLECULES OF FOCAL ADHESION AND CELL MOTILITY

CD44 molecule belongs to a family of integral membrane proteoglycans and glycoproteins involved in cell adhesion and motility (114). Aberrant mRNA splicing for CD44 has been found in PTC, whereby specific patterns of aberrant splicing may influence PTC biological behavior (115) .

β catenin (encoded by *CTNNB1)* and **E-cadherin** bind together to form adherens junctions. Mutations of *CTNNB1* have been reported in up to 20% PDTC and 70% ATC (57), with lower expression of β-catenin in DTC compared to A TC (2). On the contrary, E-cadherin expression was reported high in normal thyroid tissue and practically lost in ATC (116).

EPIGENETIC ALTERATIONS IN THYROID TUMORS

The most frequently reported epigenetic mechanisms of gene silencing and dedifferentiation in thyroid cancer has been aberrant methylation of gene promoter regions. Alterations in methylation patterns of CpG islands have been found in gene promoters of normal thyrocyte function (e.g. for Na/I symporter or thyroid-stimulating hormone receptor) and in relationship to BRAF mutation-driven MAPK signaling (60). Alternative mechanism is increased methylation of promoter with silencing of various tumor suppressor genes; examples are methylation of *PTEN* in PI3K/AKT pathway, mutations in *RAS* and mutations and amplifications of *PIK3CA* (117). In addition to DNA methylation, posttranslational histone modifications in terms of methylation, acetylation, phosphorylation and ubiquitination have been reported (118).

PROGRESS IN GENOME SEQUENCING TECHNOLOGIES

In 1977, Sanger introduced the concept of DNA sequencing in a seminal paper on chain-terminating sequencing technique based on dideoxynucleotide analogues (119). In the same year Maxam and Gilbert published chemical degradation sequencing technique based on chemical cleavage at specific bases (120). Sanger method ultimately prevailed as the basis for future refinements due to higher efficiency and utilization of fewer toxic chemicals and lower amount of radioactivity. Over the next two decades, automatization of DNA sequencers (121,122) led to steady improvement in sequencing efficiency. Sanger sequencing (the "first generation") has been considered the gold standard of sequencing due to production of the longest reads (500 bp to 1kb) and well defined chemistry, with ultimately high sequencing accuracy (123). Nevertheless, low throughput, slow speed and high costs have limited the widespread use of Sanger sequencing (e.g. the Human Genome Project spanned over a decade with estimated costs of 0.5–1 billion USD) (124).

The major milestone in sequencing technologies was achieved with the introduction of next-generation sequencing (NGS), i.e. "second generation", over a decade ago. Significant improvement of cost-efficiency in NGS was possible due to simultaneous detection of a high number of parallel sequencing reactions (125). Despite diversity of NGS techniques that evolved over the years (e.g. "454", Illumina, SOLiD, Ion Torrent), they all share a common workflow: 1. sample acquisition, 2. library formation by generation of templates (with or without amplification, depending on platform sensitivity), 3. sequencing and detection and 4. data analysis (123,126) (***Figure 5)***.

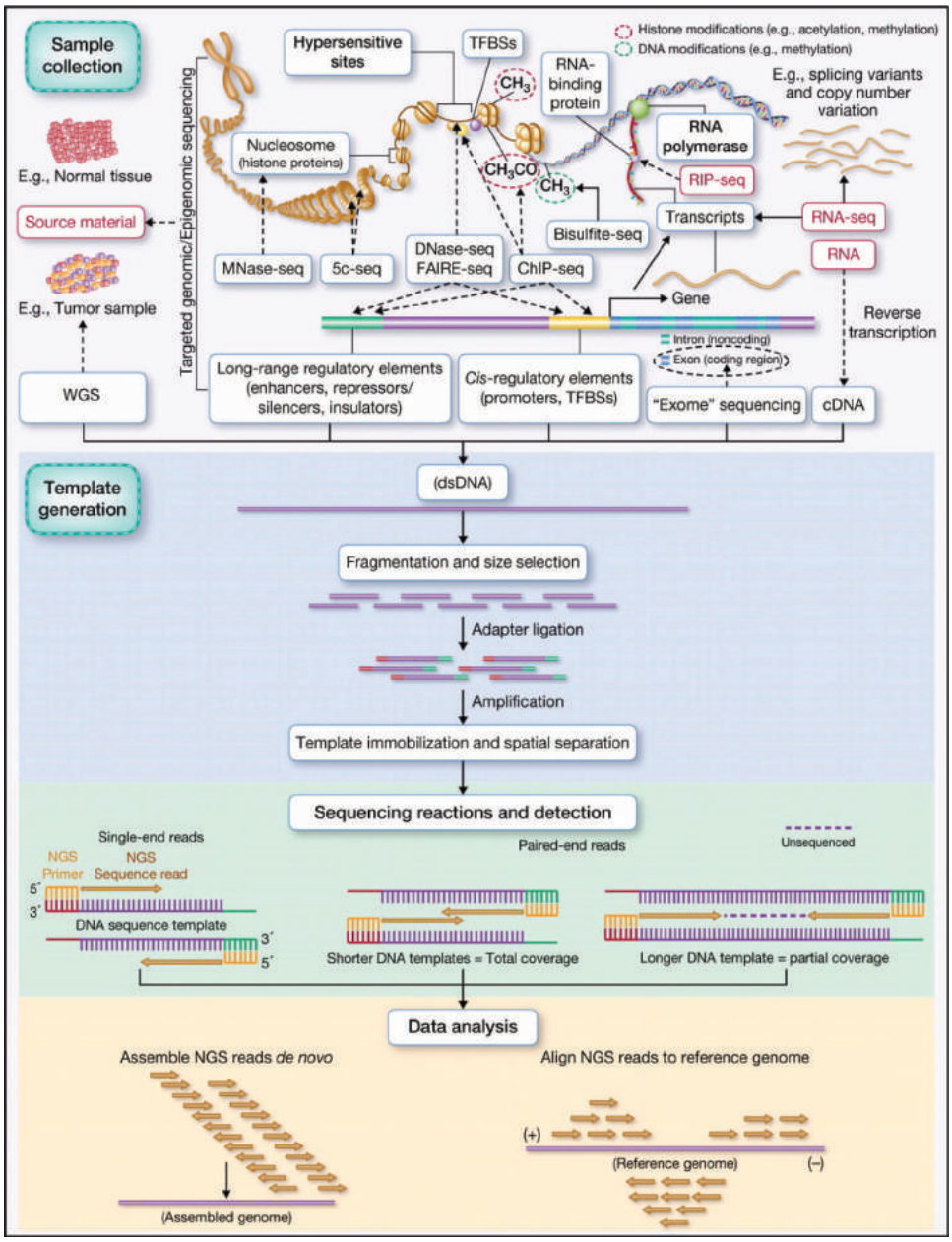

Figure 5 | Basic workflow of NGS experiments*

**reprinted from Cancer Prev Res (Phila), Volume 5/Issue 7, Page 888, Jason M. Rizzo and Michael J. Buck, Key Principles and Clinical Applications of "Next-Generation" DNA Sequencing, with permission from AACR*

One of the most adopted NGS systems worldwide has been Illumina sequencing technology due to high throughput (e.g. for Illumina HiSeq series: 1500 Gb) and relatively quick turnaround times (for HiSeq 2500: under 6 days) (127). Read length in Illumina is relatively low due to technology limitations (e.g. for HiSeq 2500 rapid run series 2X250 nucleotides) (128). However, the error rate across Illumina platforms is less than 1% (124). In our studies we performed genomic profiling on Illumina HiSeq 2500 system (rapid-run mode) (129).

AIMS AND BRIEF OUTLINE OF THE THESIS

PDTC is a separate category of follicular cell-derived thyroid cancer that has intermediate character between DTC and ATC. Reports on PDTC are lacking because of the relatively rare incidence of PDTC. Therefore studies on PDTC mandate accruing patients over longer period of time in a tertiary care center with a high volume of thyroid cancer cases. In addition, heterogeneous inclusion criteria have been employed to diagnose PDTC which often hindered comparison between studies as well as drawing conclusions about PDTC biology. Elucidating PDTC biology is important because PDTC is the most frequent cause of morbidity and death from non-anaplastic follicular cell-derived thyroid cancer. Our aim was to study one of the largest cohorts of PDTC reported in the literature, treated surgically with or without adjuvant therapy at Memorial Sloan Kettering Cancer Center (MSKCC) during a period of over 24 years. All our PDTC patients were diagnosed by homogenous criteria of proliferative grading such as mitosis and necrosis which were shown to define biologically more homogenous group of PDTC patients as opposed to growth pattern or cell type. Our main objectives were:

1. Comprehensive characterization of PDTC through study of clinical, histopathological, biochemical and genomic characteristics of PDTC patients
2. Correlation of clinical, pathological and biochemical characteristics with morbidity and mortality in PDTC patients
3. Determination of patterns of treatment failure in PDTC patients
4. Correlation of genomic profile with clinical and pathological characteristics and outcomes in PDTC patients

We believe that this comprehensive characterization of PDTC will contribute to better understanding of PDTC biology which in turn will help develop standardized clinical guidelines for diagnosis and management of PDTC. In addition, in the era of precision medicine a better understanding of PDTC biology will expedite development of novel targeted treatments, much needed in order to improve the therapeutic outcomes.

In **Chapter 2** we report on patient, tumor and treatment characteristics of PDTC patients that presented with gross ETE (T4a stage). We discuss the role of treatment and its impact on recurrence patterns and survival. In **Chapter 3** we studied all PDTC patients who were treated in a single tertiary care cancer center (MSKCC) over a twenty-four year period (1986-2009). We report on patient, tumor and treatment characteristics of PDTC, patterns of recurrence, causes of disease specific deaths, significant predictors of disease specific deaths and significant differences between PDTC patients that died of the disease versus those who survived the disease. **Chapter 4** reports a clinicopathologic study on all patients with non-anaplastic follicular cell-derived thyroid cancer who died of the disease after previously received treatment at MSKCC during 1985-2010. The study places special focus on PDTC as the most common cause of death from non-anaplastic thyroid cancer. In **Chapter 5** we report on undetectable Tg after surgery in papillary thyroid cancer (PTC) patients stratified according to GAMES and ATA criteria. We report whether undetectable Tg in those patients predicts low risk of recurrence and therefore obviates the need for adjuvant RAI. In **Chapter 6** we report on undetectable Tg after surgery and adjuvant RAI in PDTC patients with M0 and whether undetectable Tg in those patients predicts low risk of recurrence. In **Chapter 7** we examine for the first time the prevalence of *TERT* promoter mutations in thyroid cancer. In **Chapter 8** we report genomic and transcriptomic profile of PDTC and analyze our results in the context of ATC profile (sequenced simultaneously with PDTC) and in the context of the PTC TCGA study. In **Chapter 9** we report the genomic profile and clinicopathological features of fatal cases of non-anaplastic thyroid cancer (FNAT) and analyze our results in the context of PTC TCGA study and in the context of deep sequencing studies of ATC we reported previously.

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Part I

Clinical Characteristics and Outcomes in Poorly Differentiated Thyroid Cancer

Chapter 2

Poorly Differentiated Thyroid Carcinoma Presenting with Gross Extrathyroidal Extension: 1986–2009 Memorial Sloan-Kettering Cancer Center Experience

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Thyroid 2013; 23(8):997–1002

Abstract

Purpose

To describe the outcome of patients with poorly differentiated thyroid cancer (PDTC) presenting with gross extrathyroidal extension (ETE).

Materials and Methods

After obtaining Institutional Review Board approval, we performed a retrospective review of a consecutive series of thyroid cancer patients treated by primary surgical resection with or without adjuvant therapy at Memorial Sloan-Kettering Cancer Center from 1986 to 2009. Out of 91 PDTC patients, 27 (30%) had gross ETE (T4a), and they formed the basis of our study. Of 27 patients, 52% were women. The median age was 70 years (range 27–87 years). Ten patients (37%) presented with distant metastases; four to bone, three to lung, and three to both bone and lung. All patients had extended total thyroidectomy, except two who had subtotal thyroidectomy. Twenty patients (74%) had central compartment neck dissection and 11 also had lateral neck dissection. Four patients had pN0, six (30%) pN1a, and 10 (50%) pN1b neck disease. Twenty-one patients (77%) had adjuvant therapy: 15 (55%) radioactive iodine (RAI) only, three (11%) postoperative external beam radiation (EBRT) only, and three (11%) had both RAI and EBRT. Overall survival (OS), disease-specific survival (DSS), local recurrence–free survival (LRFS), and regional recurrence–free survival (RRFS) were calculated by the Kaplan Meier method.

Results

The median follow-up time was 57 months (range 1–197 months). The 5 year OS and DSS were 47% and 49%, respectively. This poor outcome was due to distant metastatic disease; 10 patients had distant metastases at presentation and a further six developed distant metastases during follow-up. Locoregional control was good with 5-year LRFS and RRFS of 70% and 62%, respectively. Overall, eight patients (30%) had recurrences: two had distant alone, two regional, two regional and distant, one local and distant, and one had local, regional, and distant recurrence.

Conclusions

Aggressive surgery in patients with PDTC showing gross ETE resulted in satisfactory locoregional control. Due to the small proportion of patients who received EBRT (22%), it is not possible to analyze its benefit on locoregional control. Of significance is the observation that the majority of patients (60%) who presented with or subsequently developed distant metastases eventually died of distant disease. New systemic therapies to target distant metastatic disease are required for improvements in outcome.

Introduction

Poorly differentiated thyroid carcinoma (PDTC) has an aggressive behavior in the spectrum of thyroid carcinoma. Although it has higher recurrence and mortality rates compared with well-differentiated thyroid carcinoma (WDTC), it is not as lethal as anaplastic carcinoma (AC). Detailed studies on PDTC are lacking due to its relatively rare incidence and controversy regarding the diagnostic criteria that define PDTC. It has only recently been recognized as a separate entity and is now defined by architectural and high-grade features according to the 2004 World Health Organization (WHO) classification (1) and 2006 Turin proposal (2). Further diagnostic criteria based on mitosis and necrosis as reported by Hiltzik et al. (3) has defined PDTC as a more homogenous and more aggressive type of tumor than the definition based on growth pattern.

PDTC tends to present with higher rates of gross extrathyroidal extension (ETE) than differentiated thyroid carcinoma (DTC) (4). In DTC, the presence of gross ETE is the most important tumor characteristic determining outcome along with patient age. Complete surgical removal of all gross disease is the key to a favorable outcome in DTC, and up to 100% 15-year survival rates have been reported (5). In PDTC, however, there is a lack of reports in the literature on the management and outcome of patients with gross ETE. The role of aggressive surgical resection has not been as clearly defined in PDTC as in DTC. The objective of our study was therefore to define the role of aggressive surgical resection on locoregional control and survival in patients with PDTC with gross ETE. We also wanted to study the patterns of treatment failure and report long-term outcome in these patients.

Materials and Methods

Following Institutional Review Board approval, a database search was made of the term PDTC for patients treated by primary surgical resection with or without adjuvant therapy at Memorial Sloan-Kettering Cancer Center (MSKCC) from 1986 to 2009. This produced 72 PDTC patients. Archived histopathology slides were available for 69 patients and were reviewed by two independent pathologists that subspecialized in thyroid malignancy (R.A.G. and D.L.C.) and were blinded to clinical data and outcome. The diagnosis of PDTC (MSKCC) was based on histologic and/or immuno-histochemical evidence of follicular cell differentiation and presence of tumor necrosis and/ or ≥ 5 mitoses per 10 high-power fields (x400) (3). Concordance rate between the two reviewing pathologists was 78% and for the rest a consensus diagnosis was reached. Of 69 PDTC cases, 57 were classified as PDTC by the MSKCC criteria, seven as DTC, three as anaplastic cancer, and two as metastatic cancer to the thyroid (lung and adenoid cystic carcinoma). In addition, the pathology of all DTC patients from 1986 to 2009 that died of the disease was also reviewed. This identified 36 further patients with PDTC (MSKCC). The total number of PDTC (MSKCC) patients was 93. Two patients were subsequently excluded: one with inoperable disease, and one with unknown primary site and PDTC in metastatic lymph nodes only. Therefore, the final number of PDTC (MSKCC) patients included in the analysis was 91. Of these, 27 patients (30%) had gross ETE with invasion of adjacent structures (T4a) at presentation based on operative and clinical reports. Staging was classified according to the AJCC Cancer Staging Manual 7th edition (6). These 27 patients form the basis of our analysis. Patient charts were reviewed, and patient characteristics, clinical presentation, treatment, tumor pathological features, recurrences, and survival were recorded. Recurrence was defined as any local, regional, or distant recurrence that was either biopsy-proven or a new radiologic finding identified on computed tomography, magnetic resonance imaging, positron emission tomography (PET) scanning, or by new radioactive iodine (RAI) uptake found at a distant site or in the neck with adequate thyrotropin stimulation. Patients were considered to have died of the disease if it was confirmed by a death certificate or a hospital summary. Overall survival (OS), disease-specific survival (DSS), local recurrence–free

survival (LRFS), and regional recurrence–free survival (RRFS) were calculated by the Kaplan Meier method and univariate comparisons between groups were done using the logrank test. Statistical analysis was carried out using commercially available software (JMP version 5.0; SAS Institute Inc., Cary, NC). Given the small sample size, multivariate analysis of outcomes was not possible. All percentages were rounded to the nearest integer.

Results

Patient and tumor characteristics are shown in Table 1. Of 27 patients, 14 (52%) were women. The median age was 70 years (range 27–87). The majority of patients (96%) were 45 years of age and older. Only four patients (15%) had a history of radiation exposure. Fourteen (52%) had a primary tumor that was larger than 4 cm. Twenty patients (74%) underwent neck dissection: all patients had a central compartment neck dissection and 11 patients also had a lateral neck dissection. Four (20%) patients had pN0 and 16 (80%) were pN +: 10 (50%) pN1b and six (30%) pN1a. The surgical margins were reported to be positive on pathology examination in 24 patients (89%). Ten patients (37%) had distant metastases at presentation: four with bone, three with lung, and three with both lung and bone metastases.

Table 1 | Patient and Tumor Characteristics

Age	
<45 years	1 (4%)
≥45 years	26 (96%)
Sex	
Female	14 (52%)
Male	13 (48%)
Radiation exposure	
Yes	4 (15%)
No	22 (81%)
Unknown	1 (4%)
Pathological size of primary tumor	
≤4cm	12 (44%)
>4cm	14 (52%)
Unknown	1 (4%)
Margin status	
Negative	2 (7%)
Positive/close	24 (89%)
Unknown	1 (4%)
Pathological neck status (*n* = 20)	
N0	4 (20%)
N1a	6 (30%)
N1b	10 (50%)
M1	
Yes	10 (37%)
No	17 (63%)

M1, distant metastasis at presentation.

Treatment details are shown in Table 2. All patients had an extended total thyroidectomy, except for two who had a subtotal thyroidectomy: in one patient the contralateral lobe was atrophic and was left in situ to be treated with postoperative radioiodine (RAI), and in another patient a small portion of the contralateral normal-appearing lobe was left in situ at the discretion of the operating surgeon. Structures that were most commonly invaded by the primary tumor were upper respiratory tract (67% of patients: five with larynx,

five with trachea, and eight with both larynx and trachea involvement), recurrent laryngeal nerve (63%) esophagus (56%), and major veins (11%). Of 27 patients, 19 had more than one structure invaded. Recurrent laryngeal nerve resection, submucosal esophagectomy (excision of the muscular wall of esophagus) and shaving the tumor off the upper airway cartilage with or without cricothyroid muscle resection were the most common types of extended procedures. Gross residual disease was left in five (19%) patients according to the operating surgeon's note. Adjuvant therapy was given in 21 patients (77%): 15 (55%) had RAI alone, three (11%) had postoperative external beam radiation (EBRT) alone, and three (11%) had both RAI and EBRT. Of five patients with gross residual disease two patients had only RAI (one had a locoregional recurrence), one had only EBRT (no locoregional recurrence), one had both RAI and EBRT (no locoregional recurrence), and one had no adjuvant therapy after refusing EBRT (the patient had a locoregional recurrence).

Table 2 | Treatment Characteristics

Structures invaded	
Upper respiratory tract	18 (67%)
Recurrent nerve	17 (63%)
Esophagus	15 (56%)
Veins, major	3 (11%)
Surgical procedures	
Shaved off trachea/larynx	11 (41%)
Cricothyroid muscle resection	6 (22%)
Tracheal window resection	0 (0%)
Tracheal segmental resection	3 (11%)
Laryngopharyngectomy	1 (4%)
Esophageal submucosal resection	14 (52%)
Esophageal total resection	1 (4%)
Recurrent nerve unilateral resection	17 (63%)
Neck dissections	
None	7 (26%)
Central compartment alone	9 (33%)
Lateral neck alone	0 (0%)
Central compartment and lateral neck	11 (41%)
Gross residual disease	
Yes	5 (19%)
No	20 (74%)
Not known	2 (7%)
Adjuvant therapy	
RAI only	15 (55%)
EBRT only	3 (11%)
RAI and EBRT	3 (11%)
Not known	1 (4%)
None	5 (19%)

RAI, radioiodine; EBRT, postoperative radiotherapy.

Of 19 patients who had only microscopic positive margins, 12 patients had only RAI (five had a locoregional recurrence), two had only EBRT (one had a locoregional recurrence), and two had both RAI and EBRT (no locoregional recurrence). No adjuvant therapy was recommended in two patients (one of them had a locoregional recurrence), and for one the status of adjuvant therapy was not known (the patient had locoregional recurrence). Using Fishers exact test, no statistically significant association was found between EBRT and loco-regional recurrence. Systemic therapy (sorafenib followed by doxorubicin) was given to 1 out of 16 patients with distant disease for palliation of RAI-refractory progressive disease. Of 27 patients, 19 patients

underwent RAI scanning: 11 had RAI uptake outside the thyroid bed, seven did not have RAI uptake outside the thyroid bed, and for one patient data were not available. Out of 10 patients with distant metastasis at presentation (M1), seven (70%) had RAI-avid M1 disease, one (10%) had non–RAI-avid M1 disease, and two patients did not undergo RAI scanning. M1 PET-positivity was revealed in two patients, while it was negative in three and not done in five (50%) patients with M1 disease.

With a median follow-up of 57 months (range 1–197 months), the 5-year OS and DSS were 47% and 49%, respectively (Fig. 1). Overall, eight patients (30%) had recurrences: two (7%) distant alone, two (7%) regional alone, two (7%) regional and distant, one (4%) local and distant, and one (4%) local, regional, and distant. Ten (37%) patients had persistent distant metastatic disease since initial presentation. The 5-year LRFS was 70% and 5-year RRFS survival was 62%. The 5 year locoregional recurrence–free survival was 56% (Fig. 2). Of five patients who had gross residual disease, two patients had no locoregional control (one had adjuvant RAI and one patient had no adjuvant treatment) and three patients had locoregional control (one had adjuvant RAI, one EBRT, and one RAI/EBRT). Of 19 patients with only microscopic residual disease (positive margins), eight had no locoregional control (five had adjuvant RAI, one had EBRT, one no adjuvant treatment, and for one data on adjuvant treatment were not available). Of 14 patients (52%) who died of thyroid cancer, 11 patients died of distant disease and three patients died of uncontrollable locoregional disease with distant metastatic disease also being present. The low survival was therefore attributable to distant metastatic disease in the majority of patients, rather than locoregional recurrence.

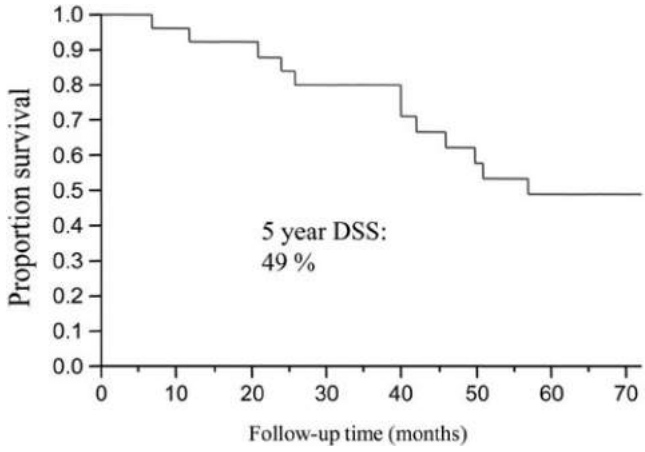

Figure 1 | Disease-specific survival in poorly differentiated thyroid cancer (PDTC) with extrathyroidal extension (ETE). Median survival time: 57 months.

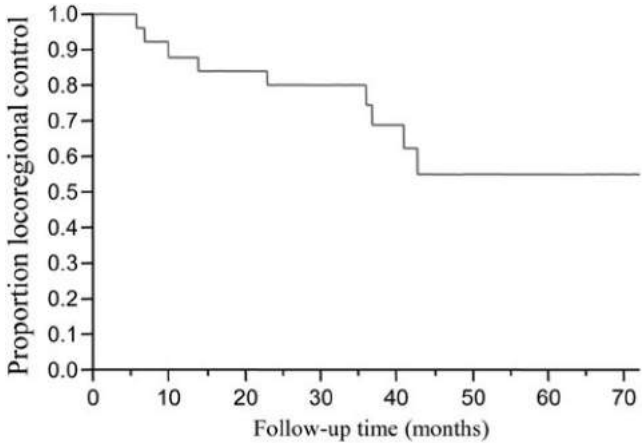

Figure 2 | Loco-regional control in PDTC with ETE. 5 year loco-regional control: 56%.

Discussion

PDTC has recently been recognized as a separate entity in the WHO Classification of Endocrine Tumors in 2004 (1) and the Turin proposal (2) based on architecture and high-grade features. It has also been reported that high-grade features such as mitoses, necrosis, nuclear pleomorphism, and invasiveness are of diagnostic and prognostic significance (7,8) and that a more homogenous group of PDTC tumors may be defined if the diagnosis is based on mitosis and necrosis rather than on growth pattern (3). PDTC has an intermediate aggressive behavior in the progression spectrum from DTC to AC. It has a tendency to present with higher rates of local invasion than DTC (4). Although there are literature reports on the outcome of patients with DTC with gross ETE (9,10), clinical studies on PDTC are limited due to its rarity and the heterogeneity of criteria for the diagnosis of PDTC. In DTC, management consists of an aggressive surgical approach in which the goal of surgery is to achieve removal of all gross disease. Indeed high survival rates can be achieved if this aggressive surgical approach is adopted (5). Our policy in the management of patients with PDTC and gross ETE is similar to that of DTC. The goal is to achieve removal of all gross disease in order to control the central compartment of the neck, minimize the risk of locoregional recurrence and hence prevent life-threatening airway obstruction or hemorrhage. However, the efficacy of this type of surgical approach in PDTC has not been reported before. The objective of our study was therefore to assess the efficacy of this approach of gross total surgical resection in the management of PDTC with gross ETE.

PDTC (MSKCC) was relatively rare among our thyroid carcinoma patients (91 patients over a period of 24 years), and this is in agreement with the relatively rare incidence reported in the literature (11). Our data show higher rates of gross ETE at presentation in PDTC (30%) than is reported for DTC, 4%(12) to 11% (13), and this supports the finding that PDTC presents with higher rates of local invasion than DTC.

The most common central compartment structures invaded in our PDTC (MSKCC) patients were similar to those reported for DTC with gross ETE. Upper respiratory tract invasion occurred in 67% of our cohort compared with 49% (9) and 82%(14) in reports on DTC. However, there was a higher frequency of recurrent laryngeal nerve involvement with 63% of our patients compared with 47% (9) and 32.6% (15) reported for DTC. In addition, there was significantly higher esophageal invasion, with 56% of our patients having invasion compared with 21% (9) and 8.6% (15) in DTC. The tendency of PDTC in our MSKCC cohort to invade more posteriorly located structures (esophagus, trachea or larynx, and recurrent nerve) compared with DTC can

be explained by the more aggressive local behavior of PDTC but also by the fact that over half of patients presented with a primary tumor larger than 4 cm in size.

The majority of our patients had extended thyroidectomy with resection of involved structures. Two patients underwent subtotal thyroidectomy, in one case to preserve a functioning contralateral recurrent laryngeal nerve due to sacrifice of the ipsilateral nerve during tumor removal, and in the second case a small portion of the contralateral lobe was left at the discretion of the operating surgeon. Removal of extrathyroid disease included organ preservation in the majority of cases: either by shaving the tumor off the trachea, larynx, or both (73% of patients with laryngotracheal invasion) or by submucosal esophagectomy (93% of patients with esophageal invasion). Partial thickness esophagectomy preserving mucosal continuity is oncologically safe in the absence of direct invasion of the mucosa because locally invading thyroid cancer is usually confined to the muscularis layer without extension into the submucosa and mucosa (16). This high rate of surgical gross disease clearance in 74% of our patients is higher than the 56% previously reported by other investigators for locally invasive DTC (9).

However, it should be noted that all 27 patients were considered operable, and patients with T4b tumors (unresectable disease) were excluded from our analysis. In the study on papillary thyroid cancer with local invasion reported by McCaffrey et al. (9), 30% of the patients had invasion of the jugular vein, carotid artery, or prevertebral fascia, with the latter two designating T4b unresectable disease. Therefore a comparison of our clearance rate with reported rates in the literature has to be interpreted with caution.

Although we had a high rate of gross disease removal, the margins of surgical resection were reported histologically close or positive in 89% of our patients. Due to the large number of patients with positive margins, the majority of our patients (77%) received postoperative adjuvant therapy (RAI, EBRT, or both). The decision for adjuvant treatment was determined by the treating physician and an individual risk estimation of the extent of gross or microscopic residual disease. However, it was also based on patient compliance, particularly in the case of EBRT. In our analysis, we did not find a statistically significant association between EBRT and locoregional recurrence. However, the patient numbers were small and therefore no conclusions can be drawn with regards to the benefits of EBRT. In general, due to the reduced morbidity associated with radiotherapy given by intensity-modulated radiation therapy, EBRT is now currently recommended more frequently and is better tolerated.

Based on the aggressive surgical approach, the 5-year locoregional control in our patients was satisfactory at 56%. The 5-year local and regional control rates (70% and 62%, respectively) were inferior to local (92%) and regional (72%) control rates reported for DTC patients with ETE (17). Despite locoregional recurrence in our patients, the reason for death was usually distant metastatic disease rather than uncontrolled central compartment disease. Only three patients died as a result of airway involvement from locoregional recurrence.

The M1 rates in our cohort (37%) were similar to M1 rates reported by other investigators in PDTC where the majority of patients presented with gross ETE (T4) (18). However, the incidence of subsequent distant metastases was lower in our cohort, 22% vs. 50% (18). In contrast, our M1 rates were higher than those reported for DTC with ETE, 37% vs. 9% (19), as were the rates of subsequent distant metastases, 22% vs. 6%(12). This indicates that distant metastatic disease is more frequent for patients with PDTC compared with DTC (4). The majority of our patients who presented with M1 disease (7 of 10, 70%) had RAI-avid disease. This is comparable to 67% RAI-avid M1 disease in DTC (20). However, it should be noted that DTC is often a much more RAI-avid disease than PDTC, and is identified both on the diagnostic and post-therapy scans. In contrast, PDTC usually shows uptake only after a large therapeutic dose. PDTC is therefore technically RAI avid but probably below the therapeutic threshold. Although patients with RAI-avid metastases have better prognosis

in DTC (21), and RAI avidity of M1 disease has a significant impact on DSS (22), it was not possible to assess the significance of RAI avidity and outcome in PDTC due to the small number of patients.

Five-year OS rates for other reports of PDTC with ETE are similar to our cohort, 47% vs. 55% (23). In contrast, survival rates reported for DTC with gross ETE are higher than in our cohort of patients with PDTC (MSKCC), 5-year OS of 79% (9) and 10-year DSS of 90.6% (10). This is due to the more aggressive biology of PDTC, with a higher incidence of distant metastatic disease compared with DTC. The cause of death in the majority of our patients was distant metastatic disease rather than uncontrolled locoregional disease. Of 14 patients (52%) who died, 11 patients died of distant disease alone, and three died of locoregional disease with distant disease also being present. In recent reports on PDTC, almost all deaths from PDTC (with 63% of patients having ETE) were caused by distant disease (50% due to distant disease, and 50% due to both local and distant disease) (18). In concordance with these results, distant disease was the only significant predictor for survival on multivariate analysis in PDTC (24).

Despite this high incidence of distant metastases, gross disease clearance in the thyroid and central compartment is still justified in these patients because it prevents death by local invasion of central compartment structures and asphyxiation. By lowering the rate of locoregional failure as a cause of death, treatment of distant disease becomes the main issue in thyroid cancer patients. Since PDTC, as it is currently defined, is a new entity, there are no standard treatments available for treating distant metastatic disease. Metastatic lesions in PDTC often do not respond to RAI due to the loss of differentiation. Metastasectomy is also rarely done in the case of PDTC. Therefore, the development of new molecular targeted therapies for PDTC is important. Most clinical trials in thyroid cancer have focused on multitargeted kinase inhibitors, which often simultaneously inhibit angiogenic targets as well as targets in the MAPK pathway (25). Furthermore, there is a potential for antiangiogenic agents to increase the efficacy of conventional therapies (chemotherapy, radiotherapy, or RAI treatment) (26). Results from clinical trials on modulators of growth, apoptosis, or other novel targets are currently pending. However, no novel treatment has yet shown improved survival for thyroid cancer patients (25). Therefore, further studies are necessary to develop targeted therapy that would result in complete response and minimal toxicity in progressive metastatic thyroid cancer, which is in most cases poorly differentiated and nonresponsive to traditional therapy.

In conclusion, we report that up to 30% of patients with PDTC (MSKCC) present with locally advanced disease with gross ETE. In these patients, up to 37% already present with distant metastases and overall over 50% will develop distant metastases. Despite this, aggressive surgical resection removing all gross locoregional disease supplemented with adjuvant therapy can produce excellent control of the disease in the neck. As a consequence, survival rates (OS and DSS) of our patients are still acceptable (47% and 49%, respectively) although poorer than those reported for DTC with gross ETE. Our study shows that while satisfactory locoregional control is achieved, the main cause of disease related death in PDTC with ETE is distant disease. Therefore, further development of novel therapies to optimally target progressive metastatic disease unresponsive to traditional modes of treatment is necessary to improve survival outcomes in this group of patients.

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Chapter 3

Outcomes in Patients With Poorly Differentiated Thyroid Carcinoma

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J Clin Endocrinol Metab 2014; 99(4):1245–1252

Abstract

Background

Poorly differentiated thyroid cancer (PDTC) accounts for only 1-15% of all thyroid cancers. Our objective is to report outcomes in a large series of patients with PDTC treated at a single tertiary care cancer center.

Methods

91 patients with primary PDTC were treated by initial surgery with or without adjuvant therapy at Memorial Sloan-Kettering Cancer Center from 1986 to 2009. Outcomes were calculated by the Kaplan-Meier method. Clinicopathological characteristics were compared for PDTC patients who died of disease to those who did not by the Chi-square test. Factors predictive of disease-specific survival (DSS) were calculated by univariate and multivariate analysis using the log rank and Cox proportional hazards method, respectively.

Results

With a median follow-up of 50 months, the 5-year overall survival and DSS were 62 and 66%, respectively. The 5-year locoregional and distant control were 81 and 59%, respectively. Of 27 disease-specific deaths, 23 (85%) were due to distant disease. Age ≥ 45 years, pathological tumor size >4 cm, extrathyroidal extension, higher pathological T stage, positive margins, and distant metastases (M1) were predictive of worse DSS on univariate analysis. Multivariate analysis showed that only pT4a stage and M1 were independent predictors of worse DSS.

Conclusions

With appropriate surgery and adjuvant therapy, excellent locoregional control can be achieved in PDTC. Disease-specific deaths occurred due to distant metastases and rarely due to uncontrolled locoregional recurrence in this series.

Introduction

Clinical studies on poorly differentiated thyroid cancer (PDTC) have been limited due to its rare occurrence (1) and heterogeneity of inclusion criteria (2–5). The World Health Organization classification (2004) (6) and Turin proposal (2006) (7) defined PDTC by a combination of architectural and high-grade features. However, further studies from our institution have indicated that high-grade features alone (mitosis and necrosis) define PDTC as a biologically more homogenous group of tumors (8). The aim of our study was to report on patient, tumor, and treatment characteristics as well as outcomes in 91 PDTC patients diagnosed on the basis of mitosis and necrosis. We previously reported on a subgroup of 27 PDTC patients with gross extrathyroidal extension (ETE) at presentation (9). In the present study, we report on a more comprehensive group of all PDTC patients diagnosed from 1986–2009, presenting the largest series of PDTC patients to date treated at Memorial Sloan-Kettering Cancer Center (MSKCC). Our aim was also to examine the difference in patient and tumor characteristics between PDTC patients that died of the disease compared to those that did not.

Materials and Methods

After Institutional Review Board approval, we carried out a database search of PDTC patients treated by primary surgery with or without adjuvant therapy at MSKCC from 1986 –2009. This search produced 72 patients. Archived histopathology slides of 69 patients were available for review by two independent pathologists (R.G. and D.L.C.) who were blinded to clinical data and outcome. There was a median of 21 slides per specimen. PDTC was defined by histological and/or immunohistochemical evidence of follicular cell differentiation and the presence of tumor necrosis and/or ≥five mitoses per 10 high-power fields (x400) (8).

Diagnosis of PDTC was confirmed in 57 of 69 patients (seven cases were reclassified as differentiated thyroid cancer [DTC], three as anaplastic thyroid cancer, and two as metastatic cancer to the thyroid [lung and adenoid cystic carcinoma]). In addition, the histopathology review of all DTC patients (1986 –2009) that died of the disease was performed, and this identified 36 additional patients with PDTC. Therefore, the total number of PDTC patients was 93. Two patients were subsequently excluded: one with inoperable disease, and one with unknown primary site and PDTC in metastatic lymph nodes only. This left 91 patients available for analysis. The concordance rate between the pathologists was 78%, and a consensus diagnosis was reached for the rest of the cases.

Patient charts were reviewed for patient characteristics, clinical presentation, tumor pathological features, treatment, recurrences, and survival. Staging was classified according to the seventh AJCC Cancer Staging Manual (10). Recurrence was defined as a new local, regional, or distant finding in a patient clinically free of the disease for 6 months after initial therapy, proven by biopsy or identified on computed tomography, magnetic resonance imaging, positron emission tomography (PET), or radioiodine (RAI) scanning. Patients were considered to have died of disease if confirmed by death certificate or hospital summary. Overall survival (OS), disease-specific survival, local recurrence- free survival, regional recurrence-free survival, and distant recurrence-free survival were calculated by the Kaplan Meier method.

Patient and tumor characteristics were compared for PDTC patients who died of the disease and those who did not by using the Pearson Chi-square test. Factors predictive of DSS were calculated by univariate and multivariate analysis by the log rank test and the Cox proportional hazards method, respectively. Probability value $P < .05$ was used to determine significance. Statistical analysis was carried out using JMP 5.0 (SAS Institute Inc) and SPSS 21.0 (IBM).

Results

Patient, tumor, and treatment characteristics (Table 1)

Of the 91 patients, 62% were female. Median age was 59 years (range, 16 –93). Sixteen patients (18%) had a history of head and neck radiation exposure.

Table 1 | Patient, Tumor, and Treatment Characteristics

	n	%
Age, y		
<45	23	25
≥45	68	75
Sex		
Males	35	38
Females	56	62
Radiation exposure		
Yes	16	18
No	66	72
Unknown	9	10
pT size, cm		
≤4	50	
>4	39	
Unknown	2	2
pT stage		
T1	4	4
T2	14	16
T3	46	50
T4a	27	30
Margin status		
Negative	35	38
Positive/close	50	55
Unknown	6	7
pN stage		
Nx	32	35
N0	22	24
N1a	14	16
N1b	22	24
N1x	1	1
M stage		
M0	67	74
M1	24	26
Type of surgery		
Total thyroidectomy	55	60
Extended total	25	28
Lobectomy	9	10
Subtotal thyroidectomy	2	2
Neck dissection		
Central	18	20
Central and lateral	23	25
LNS central	18	20
No	32	35
Gross residual disease		
Yes	5	6

	n	%
No	84	92
Unknown	2	2
Adjuvant therapy		
RAI	63	70
PORT	3	3
RAI + PORT	4	4
No	15	16
Unknown	6	7
Systemic therapy		
Yes	11	12
No	78	86
Unknown	2	2

Abbreviation: LNS, lymph nodes sampling.

Fifty patients (55%) had a primary tumor of 4 cm or less. Primary tumor staging included four (4%) pathological T stage (pT)1 patients, 14 (16%) pT2, 46 (50%) pT3, and 27 (30%) pT4a. Twenty-two patients (24%) had pathological N stage (pN)0 neck, 14 (16%) pN1a, and 22 (24%) pN1b, and one patient had no recorded location (N1x). Thirty-two patients (35%) had clinically negative neck without any neck dissection (pNx). Twenty-four patients (26%) presented with distant disease, and an additional 14 (15%) developed distant disease (17 to lung; seven to bone; 10 to lung and bone; two to skin and lung; one to lung, bone, and liver; and one to lung and brain). One patient developed bone metastasis after clearance of lung disease on computed tomography scans after RAI therapy. Eighty-eight percent of patients had total or extended total thyroidectomy, 10% had lobectomy, and 2% had subtotal thyroidectomy (one patient had an atrophic opposite lobe left in situ for RAI treatment, and another had a small portion of normal opposite lobe left in situ at the surgeon's discretion). Of the nine patients (10%) who underwent thyroid lobectomy, the reasons for lobectomy were as follows. In two patients, the ipsilateral lobe had large tumors (7.5 and 8.2 cm, respectively), and it was unclear whether the recurrent laryngeal nerve had been damaged during the resection; therefore, the surgeon decided not to remove the contralateral lobe in case the contralateral recurrent laryngeal nerve was damaged, resulting in bilateral cord paralysis necessitating tracheostomy. In one patient, the contralateral lobe was atrophic, and therefore was not removed. In the remaining six patients who had lobectomy, the initial pathology was reported as either papillary thyroid cancer or follicular cancer. These patients were subsequently diagnosed with PDTC on pathology review several years later using the current diagnostic criteria for PDTC.

Of all 91 PDTC patients, five (6%) had gross residual disease postoperatively, and an additional 45 had microscopic positive margins. At initial surgery, 18 patients (20%) had central neck dissection alone, 23 (25%) had central plus lateral neck dissection, whereas 18 patients (20%) had only intraoperative lymph node sampling for a small number (one or two) of indeterminate central compartment nodes.

Seventy patients (77%) had adjuvant therapy as follows: 63 (70%) had RAI only, three (3%) had postoperative radiotherapy (PORT) only, and four (4%) had both RAI and PORT. PORT alone was given to one patient with gross residual disease (no locoregional recurrence) and two patients with microscopic positive margins (one had locoregional recurrence). RAI and PORT were given to one patient with gross residual disease, two patients with microscopic positive margins, and one patient with no residual disease but with aggressive histopathology/extensive vascular invasion, male gender, and age 70 (none had locoregional recurrence). Using Fisher's exact test, no statistically significant association was found between PORT and locoregional control; however, having a small number of patients limits the final conclusion on the use of PORT and locoregional control.

Out of 24 patients (26%) with distant disease at presentation (M1), 17 (71%) had RAI-avid M1 disease, four (17%) had non-RAI-avid M1 disease, two (8%) did not undergo RAI treatment, and no data were available for one patient (4%). Six patients had PET-positive M1 disease, nine had PET-negative M1 disease, and no PET scans were done in nine patients with M1 (38%).

Outcomes

With a median follow-up of 50 months (range, 1–215 months), the 5-year OS and DSS were 62 and 66%, respectively (Figure 1). Twenty-four patients (26%) had persistent distant disease, and an additional 16 developed recurrences: one local and distant; two regional; four distant; two local, regional, and distant; and seven regional and distant. The 5-year local control was 90%, and 5-year regional control was 83%.

The 5-year locoregional control was 81%. Despite good locoregional control, distant control was only 59% at 5 years. Twenty-seven patients died of PDTC: 22 of distant, four of locoregional, and one of both locoregional and distant disease.

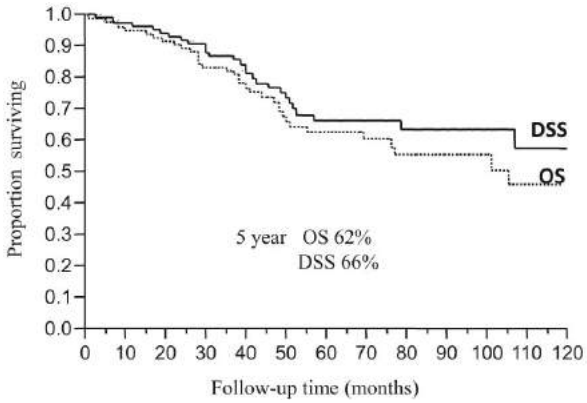

Figure 1 | Five-year OS and DSS.

Patients that died of disease vs those that survived the disease (Table 2)

Patients that died of PDTC were more likely to be older (≥45 y; 96 vs 66%; P = .002) and to present with larger tumors >4 cm; 70 vs 31%; P = .0004), ETE (89 vs 61%; P = .008), higher pT stage (T3/T4, 93 vs 75%; P = .05), and distant metastasis (M1, 59 vs 12%; P < .0001). Treatment of patients who died of the disease vs those who did not was not significantly different: total or extended total thyroidectomy (in 96 vs 84%; P= .11), neck operation (70 vs 62%; P = .47), and adjuvant radiotherapy (78 vs 76%; P = .8).

Patient and tumor factors predictive of DSS

Age ≥45 years, pT size >4 cm, higher pT, ETE, positive margins, and distant metastasis at presentation (M1) were predictive of worse outcome on univariate analysis (Table 3). Multivariate analysis of age, pT stage, and M1 showed that pT4a stage and M1 remained independent predictors of worse DSS. Patients with pT4a disease were seven times more likely to die of disease compared to those with pT1/2 disease (Figure 2A). Patients with M1 disease were three times more likely to die of disease compared to those without M1 disease (Figure 2B). pT size and ETE were not used in the multivariate analysis because they were incorporated in the pT stage variable.

Table 2 | Patient and Tumor Characteristics of PDTC Patients Who Died vs Those Who Survived the Disease

Characteristic	Patients Died of PDTC, n (%)	Patients Survived PDTC, n (%)	x^2, P Value
Age, y			
<45	1 (4)	22 (34)	
≥45	26 (96)	42 (66)	.002
Sex			
Males	14 (52)	21 (33)	
Females	13 (48)	43 (67)	.09
pT size, cm			
≤4	7 (26)	43 (67)	
>4	19 (70)	20 (31)	
Unknown	1 (4)	1 (2)	.0004
pT stage			
T1/T2	2 (7)	16 (25)	
T1	0 (0)	4 (6)	
T2	2 (7)	12 (19)	
T3/T4	25 (93)	48 (75)	
T3	10 (37)	36 (56)	
T4a	15 (56)	12 (19)	.05
ETE			
No	3 (11)	25 (39)	
Yes	24 (89)	39 (61)	
Min	3 (11)	11 (17)	
Gross	21 (78)	28 (44)	.008
Margins			
Negative	6 (22)	29 (45)	
Positive/close	18 (67)	32 (50)	
Unknown	3 (11)	3 (5)	.06
pN stage			
pNx/pN0	14 (52)	40 (63)	
Nx	8 (30)	24 (38)	
N0	6 (22)	16 (25)	
pN +	13 (48)	24 (38)	
N1a	3 (11)	11 (17)	
N1b	10 (37)	12 (19)	
N1x	0 (0)	1 (2)	.35
M stage			
M0	11 (41)	56 (88)	
M1	16 (59)	8 (12)	<.0001

Abbreviation: Min, minimal.

Table 3 | Factors Predictive of DSS

				Multivariate Analysis	
Variable	**Patients, n (%)**	**5-y DSS, %**	**Univariate Analysis, *P* Value**	**HR (CI)**	***P* Value**
Age, y					
<45	23 (25)	100			
≥45	68 (75)	60	.006	3.22 (0.389–26.81)	.278
Sex					
Males	35 (38)	54			
Females	56 (62)	77	.125	NS	NS
pT size, cm					
≤4	50 (55)	91			
>4	39 (43)	42			
Unknown	2 (2)	N/A	.0001	N/A	N/A
pT stage					
T1/T2	18 (20)	94			
T3	46 (50)	75	.0001	2.99 (0.62–14.456)	.172
T4a	27 (30)	45		6.85 (1.37–34.319)	.019
ETE					
No	28 (31)	90			
Yes	63 (69)				
Min	14 (15)	73			
Gross	49 (54)	57	.004	N/A	N/A
Margins					
Negative	35 (38)	86			
Positive/close	50 (55)	60			
Unknown	6 (7)	N/A	.02	NS	NS
pN stage					
pNx/pN0	54 (59)	70			
Nx	32 (35)				
N0	22 (24)				
pN +	37 (41)	67			
N1a	14 (16)				
N1b	22 (24)				
N1x	1 (1)		.268	NS	NS
M stage					
M0	67 (74)	85			
M1	24 (26)	34	.0001	2.97 (1.32–6.68)	.008

Abbreviations: HR, hazard ratio; CI, confidence interval; NS, nonsignificant; N/A, not applicable; Min, minimal.

Discussion

Although PDTC is rare (1, 11), it is a clinically, highly significant tumor representing the main cause of death from nonanaplastic follicular cell-derived thyroid cancer. In addition to the absence of any large series of patients with PDTC, there has been controversy with regard to the diagnostic criteria used to define PDTC. Some authors have based their diagnosis on the sole presence of solid/ trabecular/insular architecture (12). However, a relationship between tumor architecture and prognosis has not been found (13). Indeed, certain architectural patterns such as solid growth do not by themselves impart poor outcome and are not sufficient criteria for the diagnosis of PDTC (14). Moreover, it has been reported that high-grade features such as mitoses, necrosis, nuclear pleomorphism, and invasiveness provide better clinical and prognostic significance (12, 13). We have therefore used proliferative grading (high mitotic rate and/or necrosis) irrespective of growth pattern to define PDTC. PDTC cases diagnosed on the basis of these high-grade features have a worse prognosis than

those defined on architectural grounds (8). In 2007, a new approach termed the Turin proposal was published (7) and required the presence of solid/trabecular/insular architecture as well as high-grade features (tumor necrosis and/or high mitotic rate) to define this entity. Recently, Gnemmi et al (15) compared our approach to the Turin proposal and found they were complementary and similar in predicting an intermediate prognosis in thyroid carcinomas. We use proliferative grading (mitosis and necrosis) to diagnose PDTC in our practice for the following reasons: 1) it has the ability to predict intermediate prognosis validated by the study of Gnemmi et al (15); 2) it is a simple and reliable method; and 3) more importantly, PDTC defined on the basis of mitosis and necrosis constitutes the most common cause of radioactive iodine refractory fluorodeoxyglucose-PET-positive thyroid carcinoma (16), whereas no relationship was yet shown between RAI refractory disease and the other definitions of PDTC.

Using these criteria, we identified 91 patients with PDTC out of 3493 total thyroid cancer patients treated over a period of 24 years. This small number of patients illustrates the relatively rare incidence of PDTC as reported in the literature (1). Most of our PDTC patients were older, with a median age of 59 years, which corresponds to the typical age of between 55 and 63 years for PDTC patients reported in the literature (17). Likewise, 62% of our PDTC patients were females, with a female to male ratio of 1.6:1, similar to the usual 2:1 female predominance in PDTC (17).

Unlike DTCs, PDTC presents more frequently with locally invasive extrathyroidal disease (17, 18). Twenty-seven patients (30%) presented with T4a disease and 22 (24%) with gross T3 disease (gross invasion of perithyroidal fat and infrahyoid muscles, confirmed by histopathology). We have previously reported our experience on 27 patients with ETE (9), describing particular characteristics in more detail.

PDTC metastasizes to regional lymph nodes in 50 –85% of cases (1, 19), compared to 40–75% of DTC (20). However, distant metastases are much more frequent than in DTC (21–23). In our study, 38 patients (41%) had distant disease, corresponding with high rates of distant metastasis reported for other PDTC (36 – 85%) (1, 19). Similar to DTC, the most common metastatic sites are lung and bone (1, 19). All 38 of our patients with distant disease had lung and/or bone metastases. Similar to reports on DTC (24, 25), we found a strong relationship between the extent of ETE and the rate of distant metastases. Of 27 patients with gross T4a disease, 16 (59%) developed distant metastasis.

As with DTC, the extent of surgery is determined by the intraoperative findings, with the goal to clear all gross disease. Our general policy is to carry out total thyroidectomy in all cases. However, nine patients (10%) underwent thyroid lobectomy. In six of these patients, this was because PDTC was diagnosed several years later after pathological review. In the other three patients, one had an atrophic contralateral lobe, and the other two patients had very large tumors in which there was concern about the function of the ipsilateral recurrent laryngeal nerve. All nine lobectomy patients had no distant metastases at presentation (M0). Postoperatively, five of nine had pathologically positive margins, and none had gross residual disease. Three patients received adjuvant RAI (all had pathologically positive margins and one had pN1b+ neck), and one received adjuvant RAI plus PORT (due to aggressive histopathology and an advanced age with no residual disease detected). Although satisfactory locoregional control was achieved after lobectomy for unilateral intrathyroidal disease in these nine patients, two patients (22%) did die with persistent disease (locoregional + distant and distant alone). In general, if patients are diagnosed with PDTC after lobectomy, we recommend completion thyroidectomy and central compartment neck dissection.

With regard to neck management, our philosophy is to carry out central or lateral neck dissection if clinical or radiological enlargement of nodes is evident. In PDTC, regional metastases are more frequent compared to DTC, and consequently central and/or lateral neck dissection was done for 41 of our PDTC patients (45%).

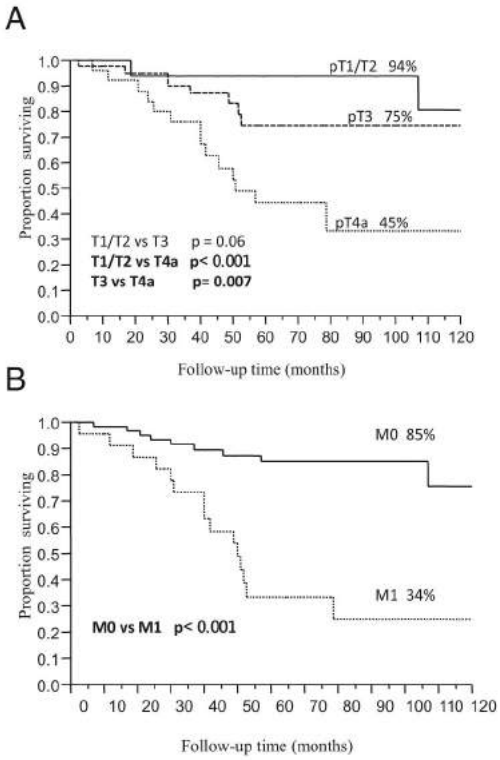

Figure 2 | 5-year DSS.

Due to the higher rate of ETE, positive margins, neck disease, and distant metastases, adjuvant treatment should be considered. Sanders et al (1) recommended considering adjuvant RAI in all PDTC patients, giving the potential benefit and lack and lack of morbidity. However, despite the capability of RAI uptake in a high percentage of PDTC, no significant impact on survival has been reported (26). Seventeen of 24 patients with M1 in our PDTC cohort (71%) had RAI-avid M1 disease. This is comparable to 67% of RAI-avid M1 disease in DTC (27). However, all 17 of our patients had persistent disease after adjuvant RAI. This is possibly explained by significant tumor heterogeneity in many PDTC, with areas of well-differentiated tumor that absorb RAI and areas of poorly differentiated tumor that do not, resulting in persistent disease despite iodine avidity. Thus, unlike in DTC (21, 22), RAI appears to be relatively ineffective in the control of distant metastases in PDTC. Although we cannot unequivocally demonstrate longer DSS or OS in those who received RAI, at present it seems reasonable to use 131-I for those distant metastases that concentrate iodine well.

PORT is even more controversial. PORT has been recommended in PDTC with T3 tumors without distant metastasis, in all patients with T4 tumors, and in all patients with neck node involvement (1). However, studies have not shown significant improvement of OS in PDTC patients after PORT (1, 26). One could argue that all 50 of our patients with pathologically positive margins (five also with gross residual disease) were candidates for PORT consideration, yet only seven patients received PORT (three PORT only, and four both RAI and PORT). Due to the small number of patients who received PORT, we did not find a statistically significant association between PORT and locoregional control, and therefore no conclusions can be drawn on the benefit of PORT. However, because reduced morbidity is now associated with intensity-modulated radiotherapy, one can argue that PORT may be more frequently recommended in patients with PDTC with positive margins or gross

residual disease. The decision, however, remains individualized, largely determined by the risk estimation of locoregional recurrence and the patients' acceptance of locoregional toxicity. In general, we now give PORT for any patient where the surgeon states that gross disease is left either at the primary site or the regional nodes. In addition, we will give PORT to select patients with positive margins. The selection is based upon the operating surgeon's opinion as to whether or not there is a suspicion for microscopic disease still being present.

Despite aggressive local and regional behavior of PDTC, the 5-year local and regional control rates in our series were excellent: 90 and 83%, respectively. This is comparable to 87% local control at 5 years in other reports on PDTC (28). Therefore, our data underscore the need for appropriate initial surgery to achieve clearance of all gross disease, resulting in 90% local control in our series.

Our PDTC had a 5-year OS of 62% and DSS of 66%. This is comparable to some reports on 5-year OS in PDTC ranging from 65–85% (5, 12, 23, 28). Univariate analysis showed that age, pT stage, ETE, margins, and M stage were significant for DSS. It was quite striking that in 23 patients under 45 years of age, only one death occurred, compared to 26 disease-specific deaths in 68 patients over 45 years of age. This indicates that even in PDTC, age remains a strong prognostic factor. However, on multivariate analysis, only pT4a and M1 remained as independent predictors of worse DSS (hazard ratios, 6.85 and 2.97, respectively). This is in agreement with other reports on PDTC reporting distant disease as a significant predictor for survival on multivariate analysis (23). Gross ETE has also been reported to adversely impact prognosis (29). Of 27 thyroid cancer-related deaths in our series, 23 (85%) were due to distant disease. Other reports on PDTC patients (where 63% had ETE) also revealed distant disease as the major cause of cancer-related deaths (30). Despite this high incidence of distant disease-related deaths in PDTC, we still strongly advocate appropriate initial surgery to obtain gross central compartment clearance to prevent morbidity and death from uncontrolled locoregional disease. Once locoregional control is obtained, treatment of distant disease becomes the main issue in PDTC patients. Because there is no standard therapy currently effective for distant disease treatment, novel molecular discoveries will most probably set directions for future therapy (17). We expect that targeting of tumor progression pathways will offer improvement in disease control and ultimately improve DSS.

Our study is not without its limitations. Most important is the retrospective nature of the study and the limitations associated with such data. Unlike DTC, the decision on the extent of surgery, the use of RAI, and the use of PORT is individualized and highly influenced by patient factors as well as the multidisciplinary team of physicians who treat such patients. Making any recommendations on treatment in such situations, especially with relatively rare diseases such as PDTC, must always be interpreted with caution. Nevertheless, our study is from an institution with over 50 years of experience in managing such patients, and as a consequence we report the largest series of such patients in the literature highlighting our management philosophy. Our study is also novel in that we report the use of our pathological grading system for PDTC as described by Hiltzik et al (8) and describe the long-term outcome of our patients based upon this system.

In conclusion, with appropriate surgery and adjuvant therapy, excellent locoregional control can be achieved in PDTC. Disease-specific deaths occur due to distant metastases and rarely due to uncontrolled locoregional recurrence in this series.

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Chapter 4

Clinicopathologic Features of Fatal Non-Anaplastic Follicular Cell–Derived Thyroid Carcinomas

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Thyroid 2016; 26(11):1588–1597

Abstract

Background

The vast majority of thyroid cancers, in particular the non-anaplastic follicular cell–derived thyroid carcinomas (non-ANA FCDC), are considered indolent tumors with very low mortality. Hence, it is crucial to analyze the subgroup of these patients who die of disease (DOD) in order to identify clinicopathologic features predictive of disease-specific mortality.

Methods

All non-ANA FCDC operated at a tertiary cancer center between 1985 and 2010 who were DOD were identified and submitted to a meticulous clinicopathologic analysis.

Results

Out of 3750 non-ANA FCDC, 58 (1.5%) DOD cases were identified. The DOD group was composed of 33 (57%) poorly differentiated carcinomas (PDTC), 14 (24%) tall-cell variant papillary thyroid carcinomas (TCVPTC), four (7%) Hu¨ rthle cell carcinomas, three (5%) papillary microcarcinomas, two (3%) classical variant PTC, and two (3%) follicular variant PTC. Twenty-seven (47%) patients presented with distant metastases (DM), 28 (48%) developed DM during follow-up, while the remaining three (5%) had locally advanced non-resectable recurrence. Gross extension beyond the thyroid (GET) was present in 36 (62%) and extensive vascular invasion (VI) in 21 (36%) of cases. All microcarcinomas had PDTC in their clinically apparent cervical lymph nodes at presentation. Encapsulated thyroid carcinomas were responsible for 17% of DOD cases, and all had extensive VI and/or DM at presentation. All patients had at least one of these high-risk features at diagnosis: DM at presentation, PDTC, GET, and/or extensive VI. The majority of patients died from DM (n = 51; 88%), three (5%) from locoregional disease, three (5%) from both, and one (2%) from unknown cause.

Conclusions

PDTC and TCVPTC are responsible for the vast majority of deaths in differentiated thyroid carcinomas, while the few fatal classical, follicular variant PTC and microcarcinomas all harbor a PDTC component, DM, or GET. Encapsulated differentiated thyroid carcinoma with focal capsular and/or VI without DM at presentation does not seem to cause death. Lack of DM at presentation, PDTC, GET, and extensive VI identify thyroid carcinomas that are at almost no risk of DOD. The vast majority of patients die of DM rather than locoregional invasion, prompting the need for effective systemic treatment.

Introduction

The incidence of thyroid cancer has been steadily increasing while mortality from thyroid cancer has gradually declined over the past few decades worldwide (1). Although reports from the 1980s and 1990s have documented a mortality rate of 3.3–11.1% in differentiated thyroid carcinomas (2–7), only 1–3% of these have been fatal in recent years (1,8). Nowadays, the vast majority of thyroid cancers, in particular the non-anaplastic follicular cell–derived thyroid carcinomas (non-ANA FCDC), are considered indolent tumors with very low mortality. Several well-recognized organizations, including the American Thyroid Association (ATA) (9) and the National Comprehensive Cancer Network (10), have published clinical management guidelines advocating for risk stratification using a variety of clinical and pathologic parameters. In order to identify subgroups at risk for poor outcome and assess the predictive power of these risk stratification schemes, it is crucial to analyze the subgroup of patients who die of disease (DOD). Toward that purpose, a meticulous clinicopathologic examination was performed of all DOD cases treated in a single tertiary cancer center with the hope that it will help better guide patient stratification and therapy.

Materials and Methods

Inclusion criteria

The study was approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY). The institutional surgical database was searched for cases meeting the following inclusion criteria: (i) patients operated between 1985 and 2010 and subsequently followed at MSKCC; (ii) a pathologic diagnosis of non-ANA FCDC, which included poorly differentiated thyroid carcinoma (PDTC), follicular carcinoma (FC), Huerthle cell carcinoma (HCC), and papillary carcinoma (PTC); and (iii) a confirmed fatal clinical outcome attributed to the thyroid carcinoma. All fatal cases were subjected to a meticulous clinicopathologic analysis under the supervision of a head and neck pathologist with special interests in thyroid neoplasia (R.G.).

Pathology review

All hematoxylin and eosin slides sampled in the specimen of the primary resection (i.e. thyroid and neck lymph nodes) were subjected to the pathologic review. Tumor size was measured as the maximum diameter of the resected tumor specimen. Mitotic rate was determined by counting 10 high-power fields (HPFs; 400x, fieldsize 0.24mm^2) with an Olympus microscope (U-DO model BX41; Olympus America, Inc., Center Valley, PA) in the areas of greatest concentration of mitotic figures. Capsular invasion was defined as complete penetration of the capsule by tumor, and the number of these foci was recorded. The presence of vascular invasion (VI) was noted only when such foci were present within or beyond the capsule in accordance with criteria outlined by the Armed Forces Institute of Pathology (AFIP) fascicle (11). Briefly, only when the invasive focus protruded into the lumen of the vessel in a polypoid manner covered by endothelial cells, or when it was attached to the vessel wall or associated with thrombus formation, was it considered true VI. Areas of VI that were closely adjacent to one another were counted as separate foci. The capsular and VI was subdivided into two categories: focal (<4 invasive foci) and extensive (≥4 foci). The presence or absence of microscopic extrathyroidal tumor extension (ETE) into the perithyroid soft-tissue stroma was documented. ETE was subdivided into (i) absent; (ii) focal (presence of one or two foci of ETE, each measuring ≤1mm); and (iii) extensive (presence of more than two microscopic foci of ETE, each measuring ≤1 mm, or any foci >1mm in size). Microscopic resection margins were categorized as positive (tumor at the inked margin) or negative (no tumor at the inked margin). Finally, when regional lymph node sampling was performed during the initial surgery, the number of lymph nodes,

metastatic status, size, presence of extranodal extension (ENE), and histological type of the metastasis were also recorded.

Poorly differentiated carcinoma was defined using the MSKCC criteria (12) and the Turin proposal (13) (Fig. 1). In brief, the MSKCC criteria defined PDTC as a non-ANA FCDC exhibiting tumor necrosis and/or an elevated mitotic index of ≥5/10 HPFs (400x), regardless of the growth pattern and nuclear features (12). On the other hand, the Turin proposal classified PDTC based on the presence of a solid/nested/insular growth pattern, the absence of nuclear features of PTC, and at least one of the following three features: convoluted nuclei, mitotic index of ≥3/10 HPFs, and/or tumoral necrosis (Fig. 1) (13).

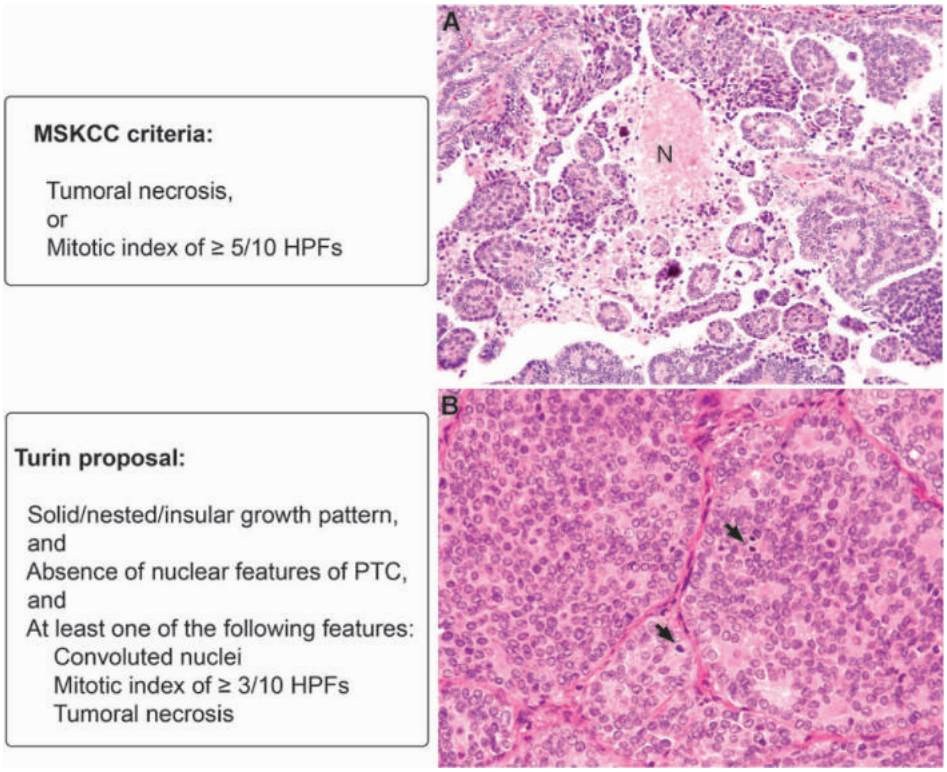

Figure 1 | Criteria to diagnose poorly differentiated thyroid carcinoma (PDTC). The Memorial Sloan Kettering Cancer Center (MSKCC) criteria require tumor necrosis or a mitotic index of ≥5/10 high power fields (HPFs). The Turin proposal requires solid/nested/insular growth pattern, and absence of nuclear features of papillary thyroid carcinoma (PTC), and at least one of the following three features: convoluted nuclei, mitotic index of ≥3/10 HPFs and/or tumoral necrosis. (**A**) Thyroid carcinoma displaying the nuclear features of PTC, as well as papillary architecture and tumor necrosis (N). This tumor will be classified as PDTC by MSKCC criteria and as PTC by the Turin proposal. (**B**) A thyroid carcinoma without the nuclear features of PTC demonstrating a solid growth pattern and a high mitotic index of 7/10 HPFs (arrows). This tumor will be classified as MSKCC-PDTC and Turin-PDTC.

Table 1 | Clinicopathologic Features of 58 Patients Harboring Fatal Non-Anaplastic Follicular Cell–Derived Thyroid Carcinomas

	N *(column %)*
N	58
Age (years), median (range)	65 (28–89)
Tumor size (cm), median (range)	4.05 (0.4–12.0)
Sex	
Female	30 (52%)
Male	28 (48%)
Classification of primary thyroid carcinoma using the MSKCC criteria for PDTC	
PDTC	33 (57%)
Hürthle cell carcinoma	4 (7%)
Tall-cell variant PTC	14 (24%)
Classical variant PTC	2 (3%)
Follicular variant PTC	2 (3%)
PTC microcarcinoma	3 (5%)
Classification of primary thyroid carcinoma using the Turin proposal criteria for PDTC	
PDTC	15 (26%)
Follicular carcinoma	1 (2%)
Hürthle cell carcinoma	6 (10%)
Tall-cell variant PTC	20 (34%)
Columnar variant PTC	4 (7%)
Solid variant PTC	1 (2%)
Classical variant PTC	5 (9%)
Follicular variant PTC	3 (5%)
PTC microcarcinoma	3 (5%)
Carcinoma of the highest histologic grade in the primary resection (thyroid and neck lymph nodes) using the MSKCC criteria for PDTC[a]	
PDTC	36 (62%)
Hürthle cell carcinoma	4 (7%)
Tall-cell variant PTC	14 (24%)
Classical variant PTC	1 (2%)
Follicular variant PTC	2 (3%)
Solid variant PTC	1 (2%)
Mitotic index (10 HPFs, 400×)	
≥5/10 HPFs	25 (43%)
0–4/10 HPFs	33 (57%)
Tumor necrosis	
Extensive	10 (17%)
Focal	11 (19%)
None	37 (64%)
Tumor encapsulation	
Partially encapsulated or non-encapsulated	47 (81%)
Encapsulated	10 (17%)
NA	1 (2%)
Capsular invasion in encapsulated carcinomas ($n = 10$)	
Extensive	6 (60%)
Focal	4 (40%)
Vascular invasion	
Extensive	21 (36%)
Focal	10 (17%)
None	25 (43%)
NA	2 (3%)

	N *(column %)*
Margin status	
Positive	29 (50%)
Negative	27 (47%)
N/A	2 (3%)
Gross extension beyond the thyroid	
Present[b]	36 (62%)
Absent	22 (38%)
Microscopic extrathyroidal extension of the dominant carcinoma	
Extensive	40 (69%)
Focal	6 (11%)
None	10 (18%)
N/A	2 (4%)
Metastasis to neck lymph nodes in patients who underwent lymph node sampling at initial surgery ($n = 44$)	
Present	35 (80%)
Absent	9 (20%)
Metastasis to ≥5 neck lymph nodes ($n = 44$)	
Present	19 (43%)
Absent	25 (57%)
ENE in 35 patients with positive lymph nodes at the initial surgery	
Present	23 (66%)
Absent	12 (34%)
Distant metastasis	
Present at initial presentation	27 (47%)
Developed during clinical follow-up	28 (48%)
Absent[c]	3 (5%)
Mode of death	
Distant metastasis	51 (88%)
Distant metastasis and locoregional recurrence	3 (5%)
Unknown	1 (2%)
Locoregional recurrence	3 (5%)

[a]*The carcinoma of the higher histological grade was documented when the histology of the carcinoma from the thyroid and from the neck lymph nodes were not identical.*

[b]*One patient with tall-cell variant PTC had gross ENE. The remaining patients had gross ETE.*

[c]*All three patients were diagnosed with tall-cell variant PTC and died due to unresectable locoregional recurrence.*

PTC, papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; HPFs, high power fields; ENE, extranodal extension; NA, not available/not applicable; ETE, extrathyroidal extension.

Clinical review

Each patient's charts were reviewed to confirm thyroid cancer–related mortality and to document the following clinical parameters: age at diagnosis, sex, type of surgery, development of locoregional recurrence and/or distant metastasis (DM), site(s) of DM, presenting symptoms at the time of initial clinical visit, time interval from initial surgery to death, and mode of demise (i.e., death related to locoregional recurrence and/or DM). Additionally, gross extension beyond the thyroid (GET), defined as any appreciable gross adhesion and/or frank invasion of the primary thyroid carcinoma or metastatic carcinoma from a neck lymph node to the adjacent structures observed during operation, was documented. Because only 15/58 (25%) of cases had post-thyroidectomy serum thyroglobulin (Tg) and thyrotropin (TSH) available in the appropriate time frame (six weeks to three months), the postoperative Tg and TSH data were not analyzed. The lack of appropriate Tg data was related to the fact that many of these cases were treated prior to 2000 when routine Tg measurements (in the appropriate time frame) were introduced.

Statistics

Statistical analyses were performed using IBM SPSS Statistics for Windows v22.0 (IBM Corporation, Armonk, NY). A two-tailed Student's t-test was performed to compare time to death between patients with DM at presentation and those without. p-values of <0.05 were considered to be statistically significant.

Results

Clinicopathologic characteristics of the study cohort

Fifty-eight (1.5%) fatal cases were retrieved from the institutional database of 3750 non-ANA FCDC. The clinical features of this cohort are summarized in Table 1. The median age at diagnosis was 65 years (range 28–89 years). There was a roughly equal sex distribution, with a male-to-female ratio of 1:1.07. The median tumor size was 4.05 cm (M = 4.4 cm; range 0.4–12.0 cm).

When MSKCC criteria of PDTC were applied, 33/58 (57%) tumors were classified as PDTC (MSKCC-PDTC). In descending order of frequency, the remaining 25 patients harbored tall-cell variant PTC (TCVPTC; n = 14; 24%), HCC (n = 4; 7%), PTC microcarcinoma variant (n = 3; 5%), classical variant PTC (CVPTC; n = 2; 3%), and follicular variant PTC (FVPTC; n = 2; 3%). Forty-four patients had lymph node sampling from the central compartment and/or lateral neck at the time of initial surgery. Metastatic carcinoma to neck lymph node(s) was detected in 35/44 (80%) patients. None of the nine patients that were pN0 (the number of lymph nodes sampled ranged from 1 to 17) at the initial surgery developed lymph node recurrence prior to their demise. Among patients with nodal metastasis, 19 patients had metastases involving at least five lymph nodes. The histologic type of the metastases was the same as the primary tumors with four exceptions. All three patients with papillary microcarcinomas harbored sizable lymph node metastasis (ranging from 1.0 to 4.7 cm in greatest diameter) in the form of metastatic MSKCC-PDTC in the lateral neck compartments (Fig. 2). Additionally, one patient with FVPTC in the thyroid harbored metastatic PTC solid variant in the neck lymph nodes. The distribution of histology in the primary resection (thyroid tumor and lymph node metastasis) was as follows: MSKCC-PDTC (n = 36; 62%), HCC (n = 4; 7%), TCVPTC (n = 14; 24%), FVPTC (n = 2; 3%), PTC classical variant (n = 1; 2%), and PTC solid variant (n = 1; 2%; Table 1).

The vast majority of the primary tumors from the study cohort demonstrated aggressive histological features, including: elevated mitotic activity (n = 25; 43%), extensive or focal tumor necrosis (n = 21; 36%), infiltrative growth pattern with no or partial tumor capsule (n = 47; 81%), extensive VI (n = 21, 36%), and extensive microscopic ETE (n = 40; 69%; Table 1). Additionally, GET causing adhesion/invasion of adjacent structure was a common finding, affecting 36/58 (62%) patients. Not surprisingly, the rate of positive surgical margins was relatively high (50%) in the cohort, possibly related to the difficulty in achieving complete resection for tumors with GET.

Fatal cases of encapsulated carcinomas and non-PDTC non-TCVPTC carcinomas

A total of 11 (19%) patients in the cohort were diagnosed with carcinomas other than PDTC and TCVPTC (Table 2), including four HCC, three papillary microcarcinomas, two CVPTC, and two FVPTC. Among them, six patients presented with DM, including one with HCC, one with papillary microcarcinoma, two with FVPTC, and two with CVPTC. The remaining five patients developed DM during clinical follow-up. Extensive VI was present in three encapsulated HCC and FVPTC. GET was documented intraoperatively in one CVPTC and in one 0.8 cm papillary microcarcinoma. Three patients were diagnosed with papillary microcarcinomas in the thyroid. However, all three patients had sizable lymph node metastasis (≥1 cm) with a pathology diagnosis of metastatic PDTC (Fig. 2). Taken together, all 11 patients with non-PDTC non-TCVPTC in the thyroid had

aggressive clinicopathologic features (e.g., extensive VI, metastatic PDTC in the lymph nodes, GET, and/or DM) at presentation.

The cohort contained 10 (17%) fatal cases of encapsulated thyroid carcinomas, including four HCC, one FVPTC, and five PDTC. All 10 cases had extensive VI (n = 8) and/or DM at presentation (n = 6; Table 3). There were two patients who did not have extensive VI, despite extensive sampling (2.0 and 2.4 tumor blocks per centimeter of tumor).

DM and mode of death

The majority of patients died from DM (n = 51; 88%), while only three (5%) died from locoregional disease and three (5%) from both (Table 1). The mode of death was unclear in one patient with advanced locoregional recurrence and extensive lung metastases. Twenty-seven (47%) patients presented with DM at the initial clinic visits, 28 (48%) developed DM during follow-up, while the remaining three (5%) had GET and positive margin at the initial resection. The median time to death was 4.1 years (range 0.2–13 years) and was significantly shorter in patients with DM at presentation (median 3.3 years; range 0.2–10.2 years) compared with those without DM (median 6.2 years; range 1.4–13 years; p = 0.001). The most common sites of DM were the lung (44/55 patients; 80%), bone (26/55 patients; 47%), and the brain (9/55 patients; 16%). Other documented sites of metastases included the liver (n = 3), adrenal gland (n = 2), pericardium (n = 1), chest wall (n = 1), pleura (n = 1), spleen (n = 1), and retroperitoneum (n = 1).

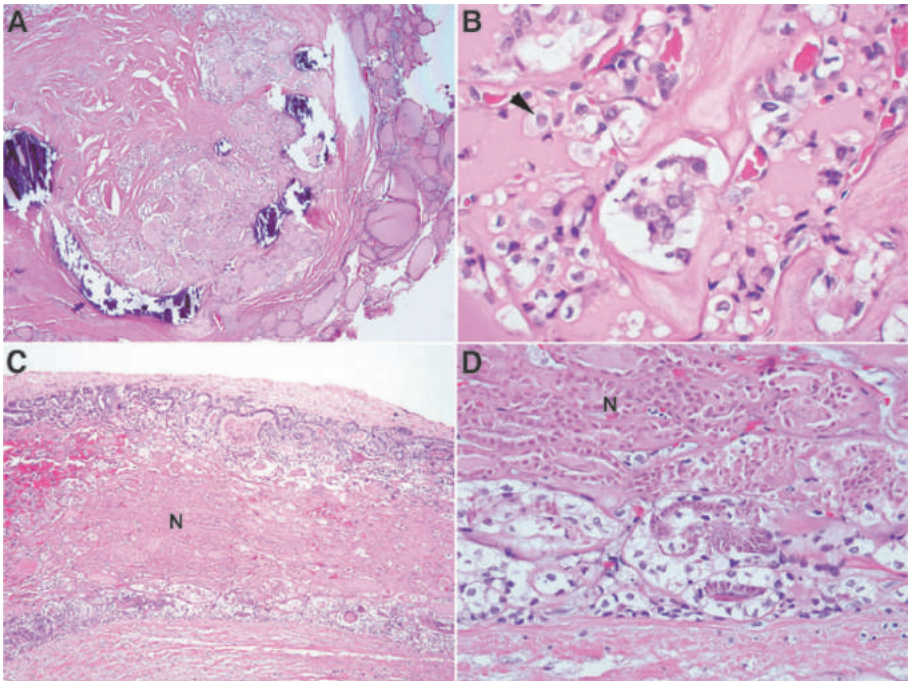

Figure 2 | A patient from the current lethal cohort with a 0.4 cm papillary micro-carcinoma of the thyroid and concurrent metastatic PDTC in a 3.2 cm ipsilateral neck level III lymph node. (**A**) The primary thyroid micro-carcinoma was a partially encapsulated papillary microcarcinoma composed exclusively of follicles. Nuclear features of papillary carcinoma, such as nuclear membrane irregularity, chromatin clearing, and nuclear grooves, were readily identified (**B**). (**C**) and (**D**) The metastatic MSKCC-PDTC in the lymph node exhibited follicular growth pattern and tumor necrosis (N) while retaining the nuclear features of PTC (**D**).

Table 2 | Clinicopathologic Characteristics of the 11 Patients Harboring Non-Poorly Differentiated, Non-Tall-Cell Variant Thyroid Carcinomas

#	*Age*	*Sex*	*Histology of primary tumor*	*Tumor size (cm)*	*Encapsulation*	*Capsular invasion*	*Vascular invasion*	*Margin status*	*Highest grade carcinoma of LN metastasis*	*ETE*	*DM*
I[a]	*AA*	M	Hurthle cell carcinoma	7.2	Complete	Extensive	Extensive	Negative	PTC, classical	Microscopic	During FU
2	75	F	Hurthle cell carcinoma	5.0	Complete	Extensive	Extensive	Negative	NA	None	During FU
3	44	M	Hurthle cell carcinoma	4.0	Complete	Focal	Extensive	Negative	NA	None	During FU
4	70	M	Hurthle cell carcinoma	5.5	Complete	Focal	None	Negative	NA	None	At presentation
5	66	M	FVPTC	5.0	Complete	Extensive	Extensive	Negative	NA	None	At presentation
6	28	F	FVPTC	2.0	None	NA	None	Positive	FVPTC	Microscopic	At presentation
7	78	F	PTC, classical	NA	None	NA	None	Positive	NA	GET	At presentation
8	76	M	PTC, classical	2.0	Partial	NA	Focal	Negative	PTC solid	Microscopic	At presentation
9	58	M	PMC	0.4	None	NA	Focal	Negative	PDTC	None	During FU
10	51	F	PMC	0.4	None	NA	None	Negative	PDTC	None	At presentation
11	77	M	PMC	0.8	Partial	NA	None	Negative	PDTC	GET	During FU

[a]This patient harbored multifocal thyroid carcinomas, including an encapsulated thyroid-confined 7.2 cm Hurthle cell carcinoma with extensive vascular and capsular invasion, an encapsulated Hurthle cell carcinoma with two foci of capsular invasion, and two PTC (one of which had microscopic extrathyroidal extension into the perithyroidal adipose tissue). The histological classification of the lymph node metastases was papillary carcinoma, classical variant.

LN, lymph node; M, male; F, female; FVPTC, follicular variant papillary thyroid carcinoma; PMC, papillary microcarcinoma; PDTC, poorly differentiated carcinoma according to MSKCC criteria; NA, not applicable; FU, follow-up; DM, distant metastasis.

Among the 27 patients who were diagnosed with DM at initial presentation, 13 patients sought clinical attentions as a result of symptoms related to DM such as hip pain (n = 4), back pain (n = 3), chest pain (n = 2), dyspnea (n = 2), palpable chest wall mass (n = 1), and cognitive alteration (n = 1). Thirteen patients presented with palpable or symptomatic locoregional disease and were found to have DM during the initial radiological investigations. The remaining patient was asymptomatic and had incidental bilateral pulmonary nodules detected during routine workups for long-standing chronic obstructive pulmonary disease. These lung deposits were subsequently proven to be metastatic thyroid carcinoma on biopsies. The histologic diagnoses of the primary thyroid carcinomas for the 27 patients with DM at presentation were as follows: PDTC (n = 17; 63%); TCVPTC (n = 4; 15%), CVPTC (n = 2; 7%), FVPTC (n = 2; 7%), PTC micro-carcinoma (n = 1; 4%), and HCC (n = 1; 4%).

Correlation between MSKCC criteria and Turin proposal in diagnosing PDTC

All patients who were diagnosed as PDTC using the Turin proposal criteria (Turin-PDTC) were also classified as PDTC using the MSKCC criteria (MSKCC-PDTC). Conversely, only 45% of PDTC classified using the MSKCC criteria fulfilled the Turin proposal definition of PDTC (Table 4). If the Turin proposal was used to define PDTC, the remaining cases would be classified as variants of PTC (n = 15; 45%), as these tumors retained some nuclear features of PTC, or less commonly as FC/HCC (n = 3; 9%), as they lacked a solid/nested growth pattern. Those 15 PTC cases would be regarded as high-grade PTC by many endocrine pathologists.

When using MKSCC criteria to define PDTC, all patients in the cohort had at least one of the four high-risk features at diagnosis: DM at presentation, GET, a pathologic diagnosis of MSKCC-PDTC in the initial resection (thyroid primary tumor and/or lymph node metastasis), or extensive VI (Fig. 3A). On the other hand, if one was to apply the Turin proposal for PDTC, 5/58 patients would be labeled as PTC without extensive VI, DM at presentation, and GET (Fig. 3B). The characteristics of these five patients are summarized in Table 5. Using the risk stratification system proposed by the ATA 2015 guidelines for differentiated thyroid carcinoma (9), one

patient would be categorized as high risk because of pathologic N1 disease, with the largest metastatic lymph node measuring 3.2 cm in the greatest dimension (patient #1); three would be classified as intermediate risk, with TCVPTC (patient #2 and #3) and columnar variant PTC (patient #4); and one would be classified as low risk (patient #5). All five patients harbored tumors without lymphovascular invasion. Although one of these low-/intermediate-risk carcinomas (patient #5) would be deemed not to require radioactive (RAI) treatment using the 2015 ATA guidelines (9), all five patients underwent total thyroidectomy and received RAI treatment at the initial presentation. Additionally, if one was to use dynamic ongoing response to therapy reclassification during the first two years of follow-up (14), two of the four low-/intermediate-risk PTC would be categorized as having a structural incomplete response (patients #2 and #4), while the remainder would be classed as having an excellent response (patient #3 and #5).

Table 3 | Clinicopathologic Features of the 10 Patients with Encapsulated Thyroid Carcinomas

#	*Age*	*Sex*	*Histology of primary tumor*	*Tumor size (cm)*	*Capsular invasion*	*Vascular invasion*	*Highest grade carcinoma in LN*	*ETE*	*DM*	*Time to death (years)*	*Mode of death*
1[a]	44	M	HCC	7.2	Extensive	Extensive	PTC, classical	Microscopic	During FU	5.8	DM
2	75	F	HCC	5.0	Extensive	Extensive	NA	None	During FU	8.2	DM
3	44	M	HCC	4.0	Focal	Extensive	NA	None	During FU	8.4	DM
4	70	M	HCC	5.5	Focal	None	NA	None	At presentation	0.2	DM
5	66	M	FVPTC	5.0	Extensive	Extensive	NA	None	At presentation	2.9	DM
6	77	M	PDTC	4.4	Focal	Focal	NA	None	At presentation	4.9	DM
7	74	F	PDTC	5	Extensive	Extensive	NA	GET	At presentation	4.8	DM
8	61	M	PDTC	8.7	Focal	Extensive	NA	GET	At presentation	4.2	DM
9	59	M	PDTC	5.3	Extensive	Extensive	NA	Microscopic	At presentation	2.5	DM
10	51	F	PDTC	4.0	Extensive	Extensive	NA	None	During FU	8.9	DM

[a]Patients #1–5 correspond to patients #1–5 in Table 2.

HCC, Hürthle cell carcinoma; GET, gross extension beyond the thyroid.

Discussion

Clinical features of fatal non-ANA FCDC

Differentiated thyroid carcinoma is considered an indolent cancer that rarely causes death. To date, only a handful of studies have been published focusing exclusively on lethal non-ANA FCDC, and most of the data came from earlier studies between 1964 and 1999 (2–6,15,16). The reported mortality is 4.2–5.4% in differentiated FCDC overall (4–6), 3.3–6.5% in PTC (2,3,5), and 11.1% in FC (2). The present study reports a considerably lower mortality rate of 1.5% from a large cohort of 3750 patients of non-ANA FCDC treated in a single tertiary cancer center. It is compatible with the 1–3%population-based mortality of thyroid cancer reported worldwide in recent years (1,8), reflecting the steady decline in mortality from thyroid cancer over the past few decades.

Table 4 | Correlation Between MSKCC Classification and Turin Proposal for PDTC

Patients with a diagnosis of PDTC using MSKCC criteria (*n* = 33)	
PDTC using the Turin proposal	15 (45%)
Well-differentiated carcinoma using Turin proposal	
Follicular carcinoma	1 (3%)
Hürthle cell carcinoma	2 (6%)
Tall-cell variant PTC	6 (18%)
Columnar variant PTC	4 (12%)
Classical variant PTC	3 (9%)
Solid variant PTC	1 (3%)
Follicular variant PTC	1 (3%)

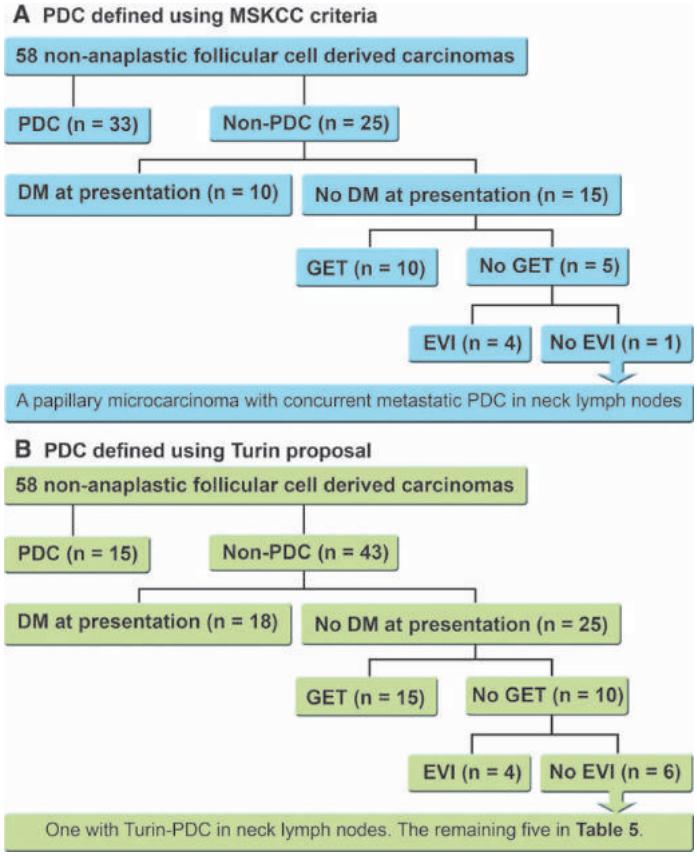

Figure 3 | Characteristics of the fatal non-anaplastic follicular cell–derived carcinomas based on four clinicopathologic features: PDTC, distant metastasis at presentation, gross extension beyond the thyroid, and extensive vascular invasion using MSKCC criteria (**A**) and the Turin proposal (**B**).

Compared with PTC and FC, which usually affects patients in their 40s (1,17), fatal non-ANA FCDC tends to occur in older patients, with a mean age at presentation of 52–59 years in previous studies (3,4,6,15) and 65 years in the present cohort. In the current study, lethal non-ANA FCDC affected females and males equally, similar to the sex ratio reported by Smith et al. (3), suggesting a higher mortality in male patients with non-ANA FCDC.

Patients who die of differentiated thyroid carcinoma commonly develop DM during their clinical course. The reported rate of DM ranges from 74% to 82.8% (3,4,6). In the present cohort, the rate was 95%. The most common sites of DM in a descending rate of involvement are lung, bone, and brain (3,4), which is confirmed by the current study. Smith et al. (3) reported that a shorter interval between initial surgery and the discovery of DM correlated with decreased survival. Similarly, in the present cohort, the median time from diagnosis to death was significantly shorter in patients with DM at initial presentation (3.3 years) compared with those without DM (6.2 years). In two studies from Japan published in the 1990s, the immediate cause of death was attributed solely to uncontrollable locoregional disease in 29% and 35.4% of patients, respectively (4,6). In contrast, in the present study, locoregional disease was the immediate cause of death in <10% of non-ANA FCDC. Such a difference could be explained in part by the advances in locoregional control in recent years (18).

Table 5 | Clinicopathologic Characteristics, Initial Risk Stratification, and Response to Therapy Reclassification in the Five Patients Classified as MSKCC-PDTC but Non-PDTC by Turin Proposal, and Lacking DM at Presentation, EVI, and GET

#	Age	Sex	Classification by Turin proposal		Tumor size (cm)	Margin status	Total number of lymph nodes with metastasis	Size of the largest lymph node (cm)	Mode of death	Time to death (years)	Site of DM	ATA 2015 risk stratification	Response to treatment
			Primary tumor	Lymph node metastasis									
1	58	M	PTC, microcarcinoma[a]	Follicular variant PTC	0.4	Negative	2	3.2	Distant	8.4	Lung, bone, retroperitoneum	High risk	Biochemical incomplete response
2	82	F	Tall-cell variant PTC	Tall-cell variant PTC	4.1	Positive	4	1.2	Distant	1.4	Lung	Intermediate risk	Structural incomplete response
3	70	F	Tall-cell variant PTC	Tall-cell variant PTC	1.8	Positive	8	0.6	Distant	11.3	Lung	Intermediate risk	Excellent response
4	78	F	Columnar variant PTC	NA	2.5	Negative	NA	NA	Distant	3.1	Lung	Intermediate risk	Structural incomplete response
5	72	F	Classical variant PTC	Classical variant PTC	2	Negative	3	0.6	Unknown	11.9	Lung	Low risk	Excellent response

[a]*This is the patient shown in Figure 2.*

ATA, American Thyroid Association.

Pathologic features that may identify potentially fatal non-ANA FCDC

The histologic composition of the lethal cohort included 57% MSKCC-PDTC, 7% HCC, and 36% PTC (including 24% TCVPTC). Alternatively, if the Turin proposal was applied to define PDTC, the cohort consisted of 26% Turin-PDTC, 2%FC, 10% HCC, and 64% PTC. Piana et al. (2010) studied 43 lethal cases using the Turin proposal for PDTC and reported similar results. In their study, 67% of fatal cases were PTC, 21% PDTC, 7% widely invasive FC, and 5% HCC (5).

All lethal carcinomas in the present cohort demonstrated at least one of four aggressive clinical or pathologic features, including DM at presentation in 47% of patients, GET appreciated during the initial surgery in 62%, a diagnosis of primary MSKCC-PDTC in 57%, and presence of extensive VI in 36%. Hence, these four features may be useful tools at the time of initial surgery to recognize potentially fatal tumors and assist subsequent clinical decision making. Importantly, the absence of these four features can be used to identify patients at an extremely low risk of death, so they can be reassured and not overtreated. Due to the study design, it is not possible to provide the frequency of these four features in nonfatal differentiated carcinomas. However, unpublished data from our group analyzing 1072 patients with differentiated thyroid carcinoma have shown that extensive VI and MSKCC-PDTC were relatively infrequent, accounting for 5% and 9% of cases, respectively.

Controversy surrounding PDTC

In the early 1980s, PDTC was first described as a group of tumors intermediate between the indolent differentiated thyroid carcinomas and the nearly always fatal anaplastic carcinoma (17). Since then, there has been continuous controversy with regard to the very definition of this entity. The MSKCC criteria (12) define PDTC based exclusively on elevated mitotic index (>5/10 HPFs) and/or tumor necrosis, regardless of tumor growth pattern and nuclear features. On the other hand, the Turin proposal for the diagnosis of PDTC is more restrictive (13). It requires the presence of insular/solid/trabecular growth pattern, the absence of nuclear features of PTC, and one or more of the following three features: convoluted nuclei, mitotic index ≥3/10 HPFs, and/or tumor necrosis. The reported mortality rates in PDTC using the MSKCC criteria and the Turin proposal have been 38% and 41%, respectively (12,13). Because the MSKCC criteria for PDTC encompasses tumors with a FC, HCC, and PTC phenotype, the rate of PDTC (57% in this cohort using the MSKCC definition) is higher than when the Turin proposal is used to classify tumors (26%). Under the Turin approach, carcinomas with high-grade features (necrosis, high mitotic rate) and a PTC nuclear phenotype are not labeled as PDTC but rather as PTC. This is an important subset, since it comprises 26% (15/58) of the cohort of fatal patients. The latter terminology may misguide the clinician into thinking he/ she is dealing with an indolent PTC. Indeed, in the current cohort of fatal cases, four carcinomas would have been categorized as low to intermediate risk according to ATA stratification if the Turin proposal was applied, as they lacked high-risk features (e.g. GET, extensive VI, DM at presentation, or pN1 disease involving lymph nodes ≥3 cm in size). Furthermore, one patient may have been treated conservatively without RAI therapy. A dynamic ongoing response to therapy reclassification would have signaled a more aggressive behavior to the clinician in some (2/4) but not all of these patients classified as low/intermediate risk by the Turin proposal. Hence, if a pathologist uses the Turin proposal to define PDTC, the term "high grade" should be used in designating these PTC in order to communicate to the clinician their potential for a fatal outcome. This will prevent false reassurance and prompt more aggressive management.

The fact that the rare PDTC (5–7% of all thyroid carcinomas) are overrepresented in fatal non-anaplastic FCDC could be explained by their genotype. In addition to the presence of a significant amount of RAS (mainly found in Turin PDTC) and BRAF mutations (mainly detected in PDTC diagnosed solely by MSKCC criteria), these PDTC display additional genetic events (18). When compared with PTC, PDTC are associated with a higher

mutation burden and a higher rate (40%) of telomerase reverse transcriptase (TERT) promoter mutations (19). The TERT promoter mutation is a molecular signature associated with aggressive clinical behavior, including a propensity for DM and disease-specific death (19,20).

Fatal papillary microcarcinoma and encapsulated thyroid carcinoma

The overall mortality of papillary microcarcinoma is extremely low, reported at 0% for incidental and 0.1% for non-incidental papillary microcarcinomas (21). However, rare fatal cases of papillary microcarcinomas have been documented in the literature (22). The ATA 2015 management guidelines have acknowledged an alternative conservative approach of active surveillance management for papillary microcarcinomas without clinical evident metastases, local invasion, and no convincing cytological or molecular evidence of aggressive disease (9). Hence, it is critical to identify papillary microcarcinomas that could be potentially fatal at the time of initial presentation in order to avoid undertreatment. Piana et al. (2013) reported three such cases of papillary microcarcinomas (22). All three patients had metastases to cervical lymph nodes at the time of initial surgery: one patient had a 6 cm lymph node containing TCVPTC, one had a 3 cm lymph node with metastatic PDTC, and one patient presented with metastatic TCVPTC with elevated mitotic index (5/10 HPFs) and ENE involving all five lymph nodes examined. These patients subsequently developed DM, which eventually led to the patients' demise. Similarly, in the present cohort, three (5%) patients were diagnosed with papillary microcarcinomas. All three had metastatic PDTC in multiple lymph nodes measuring at least 1.0 cm in greatest dimension, and all three developed DM either at the time of presentation or subsequently. The current data and that of Piana et al. clearly show that all reported fatal papillary microcarcinomas harbor certain aggressive clinico-histological features at initial presentation (e.g., PDTC transformation in the lymph node metastases, multiple/ large lymph node metastases, and/or DM at presentation). These aggressive features would disqualify these patients from all known active surveillance protocols.

Ten (17%) patients of the present fatal cohort harbored encapsulated carcinomas, including five cases of encapsulated PDTC, four encapsulated HCC, and one encapsulated FVPTC. All 10 cases inevitably exhibited extensive VI or DM at presentation. These findings are in line with what has been previously reported by Piana et al. (5) and Xu et al. (23). Encapsulated differentiated FCDC without extensive VI or DM at presentation appear to be highly indolent lesions and do not seem to cause death.

In conclusion, this study demonstrates the power of meticulous clinical, operative, and histopathologic examination in stratifying differentiated thyroid carcinoma. Based on the presence of one of four aggressive features (PDTC in the primary resection, extensive VI, GET, and/or DM at presentation), one can recognize patients at risk of death from thyroid carcinomas. The lack of any of these aggressive clinicopathologic findings identifies those harboring almost no risk of dying, thus prompting reassurance and avoiding overtreatment. The vast majority of patients die of DM rather than locoregional invasion, prompting the need for molecular analysis of these cases in order to administer and develop effective systemic treatment.

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Part II

Biochemical Characteristics of Poorly Differentiated Thyroid Cancer:

Thyroglobulin as a Disease Marker in Differentiated vs. Poorly Differentiated Thyroid Cancer

Chapter 5

Undetectable Thyroglobulin after Total Thyroidectomy in Patients with Low- and Intermediate-risk Papillary Thyroid Cancer— Is There a Need for Radioactive Iodine Therapy?

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Surgery 2012; 152(6):1096–1105

Abstract

Background

The efficacy of radioactive iodine therapy (RAI) in patients who have an undetectable thyroglobulin (Tg) level after total thyroidectomy in well-differentiated papillary thyroid cancer (PTC) is questionable. The objectives of this study were to report the risk of recurrence in patients with PTC who had an undetectable Tg level after total thyroidectomy managed with postoperative RAI and without RAI.

Methods

After approval by the institutional review board, 751 consecutive patients who had total thyroidectomy for PTC as well as postoperative Tg measurement were identified from our institutional database of 1163 patients treated for well-differentiated thyroid carcinoma at Memorial Sloan Kettering Cancer Center between 1999 and 2005. Of these, 424 patients had an undetectable postoperative Tg (defined as a Tg <1 ng/mL) of whom 80 were classified as low, 218 intermediate, and 126 high risk via use of the GAMES (grade, age, distant metastasis, extrathyroidal extension, and size of the neoplasm) criteria. Patient, neoplasm, and treatment characteristics were recorded on the low- and intermediate-risk patients. Recurrence was defined as any structural abnormality on examination or imaging and confirmed by fine-needle aspiration biopsy. Disease-specific survival and recurrence-free survival (RFS) were calculated with the Kaplan-Meier method. Univariate analysis was carried out by the log rank test and multivariate analysis by Cox proportional hazards method.

Results

In the low-risk group (n = 80), 35 patients received postoperative RAI and 45 did not. Comparison of patient and tumor characteristics showed patients treated without RAI were more likely to have T1 tumors (82% vs 60%, P = .027). There were no disease-specific deaths in either group. There was 1 neck recurrence in the group that did not receive RAI. Patients managed without RAI had a similar RFS to patients managed with RAI (96% vs 100%, P = .337). In the intermediate risk group (n = 218), 135 were managed with RAI and 83 without. Comparison of patient and tumor characteristics showed patients managed without RAI were more likely to be older patients (≥ 45 years: 90% vs 39%, P < .0005) with smaller tumors (pT1T2: 97% vs 62%, P < .0005) and negative neck disease (N0: 56% vs 30%, P <.0005). There were no disease specific deaths in either group. There were 7 recurrences, of which 6 were in the RAI cohort (5 regional, 1 distant) and 1 in the non-RAI cohort (1 regional). Patients managed without RAI had a similar RFS to patients managed with RAI (97% vs 96%, P = .234).

Conclusion

Select low- and intermediate-risk group patients who have undetectable Tg after total thyroidectomy for PTC can be managed safely without adjuvant RAI with no increase in risk of recurrence.

Introduction

Papillary thyroid carcinoma (PTC) is the thyroid malignancy encountered most frequently. There has been a substantial increase in the incidence of papillary carcinoma during the last 50 years in many countries, which is largely attributable to increased detection of small cancers (1,2); disease-specific mortality, however, has not increased during this time (3). In conjunction, there has been an increase in the number of patients undergoing total thyroidectomy and also an increase in the number of patients receiving adjuvant postoperative radioactive iodine (RAI). Recent reports have shown an association between this increased use of RAI and second primary cancers (4,5). This, together with the known complications and sequela associated with RAI (6,7), has raised some concern about the routine use of postoperative RAI, particularly in low-risk patients.

Most authors agree that low-risk patients (intraglandular tumors <1 cm) do not benefit from adjuvant RAI. However, the identification of patients who will benefit from adjuvant RAI is more controversial. Current guidelines of the American Thyroid Association (ATA) (8) recommend the administration of RAI after total thyroidectomy for differentiated thyroid cancer (DTC) in patients with known distant metastases, gross extrathyroidal extension, primary neoplasm size >4 cm even in the absence of other greater-risk features, select patients with tumors 1–4 cm confined to the thyroid who have lymph node metastases, and select patients with greater-risk tumor histology that predicts moderate to high risk of recurrence or death from thyroid cancer. Indication for adjuvant RAI in patients with an undetectable thyroglobulin (Tg) level after total thyroidectomy, which implies surgical removal of all disease and all normal thyroid tissue, has not been separately evaluated.

In our institution, we have practiced an approach of methodical "extracapsular total thyroidectomy," that is, removing all thyroid tissue with particular attention given to the superior pole, Berry's ligament, and the pyramidal lobe, together with removal of all suspicious lymph nodes (identified by palpation and/or imaging), with the aim of achieving na undetectable postoperative serum Tg. By using GAMES (grade, age, distant metastasis, extrathyroidal extension, and size of the neoplasm) criteria (9), we risk stratify patients into low, intermediate, and high risk of death (Table I). In patients categorized as low or intermediate risk, we use the postoperative Tg as a measurement of completeness of resection and then select patients who may benefit from RAI and those who can be managed without RAI with close observation. The objective of this study was to report the rate of recurrence in low- and intermediate-risk patients with PTC who had an undetectable Tg after total thyroidectomy stratified by the use of postoperative RAI. We hypothesize that undetectable postoperative Tg in otherwise low- or intermediate-risk patients with PTC identifies group of patients who can be treated without adjuvant RAI with no increase in the incidence of structural recurrence.

Materials and Methods

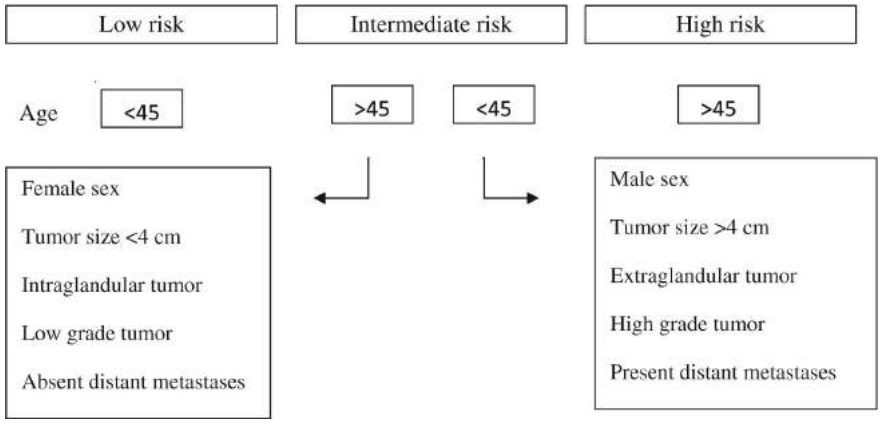
Following approval by the Institutional Review Board, 751 consecutive patients with PTC who had total thyroidectomy as well as a postoperative Tg measurement were identified from our institutional database of 1,163 patients treated for well-differentiated thyroid carcinoma at MSKCC between 1999 and 2005. Of these, 424 (56%) patients had an undetectable unstimulated Tg after total thyroidectomy. Undetectable Tg was defined as an unstimulated Tg <1 ng/mL measured 6-8 weeks after total thyroidectomy. Tg antibodies were routinely done in conjunction with the Tg assay and therefore samples with Tg antibody interference were excluded from the analysis.

All patients had a methodical total thyroidectomy, with the surgeon taking care to remove thyroid tissue from the superior pole, Berry's ligament, and pyramidal lobe. Select patients also underwent node dissection for

clinically suspicious neck nodes at the time of total thyroidectomy. In our institution we do not routinely carry out elective dissection of either the central compartment or lateral compartment lymph nodes. Central and lateral neck dissections are only performed if clinically suspicious lymph nodes are identified. In the period 1999-2005, preoperative ultrasound imaging was not used routinely for central and lateral neck compartment assessment; therefore, assessment of the neck was determined by clinical examination preoperatively and at the time of the procedure. If clinically suspicious lymph nodes were found in the lateral compartment, removal of lymph nodes in levels II-V was undertaken. If clinically suspicious lymph nodes were found in the central compartment, bilateral paratracheal lymph node dissections were carried out. In the central compartment, if a small number (1-2) of indeterminate lymph nodes were present, lymph node sampling was carried out.

Out of 424 patients with undetectable Tg, 80 patients were classified as low, 218 intermediate, and 126 high risk for death using GAMES criteria (9). GAMES criteria consider patient and tumor factors in risk stratification (Table I). This classification was designed to predict the risk of death from well-differentiated DTC, rather than the risk of recurrence. Patients are considered at low risk if younger than the age of 45 years and high risk if older than 45 years.

Table I | GAMES criteria (MSKCC) for risk of death of the disease



Tumors with more aggressive histology, which are larger than 4 cm, and/or have extrathyroid extension or distant metastases are considered high-risk tumors. In contrast, well differentiated tumors that are less than 4 cm, are intrathyroidal, and have no evidence of distant metastases are considered low-risk tumors. Thus, patients older than 45 years of age with high-risk tumors were included in the high-risk group. Patients younger than the age of 45 with low-risk tumors were included in the low-risk group. All others were included in the intermediate-risk group, that is, young patients with high-risk tumors or older patients with low-risk tumors.

For the purpose of this study, we only analyzed low and intermediate-risk patients with undetectable Tg because the most controversy on the benefit of adjuvant RAI is within these 2 groups of patients. Tumors in the high-risk group generally are treated with RAI and this is not controversial. In addition, high-risk patients are more likely to have less-differentiated tumors and tend to produce less Tg. Therefore the utility of postoperative (undetectable) Tg is much less reliable as an indicator of completeness of resection as well as avidity to RAI. For both of these reasons, we chose not to analyze the high-risk group.

Patient, tumor, and treatment characteristics were extracted on the 80 low-risk and 218 intermediate-risk patients (GAMES criteria) (9) from a preexisting database. Data included patient demographics, exposure

to radiation, and pathologic tumor-node-metastasis status. All patients had initial total thyroidectomy, and selected patients underwent neck dissection for clinically suspicious neck nodes. In GAMES-classified low-risk patients (n = 80), 10 had a central neck dissections and 19 had random lymph nodes sampling of the central compartment. All patients had pathologic negative neck disease (pN0); 51 patients had no neck node operation (pNx). In GAMES-classified intermediate-risk patients (n = 218), 60 had formal neck dissections (33 central alone, 7 lateral alone, and 20 combined central and lateral neck dissections) and 102 patients had random lymph nodes sampling. A total of 75 patients had pathological positive neck disease (pN+); 56 patients had no neck node operation (pNx).

Details of RAI administration were collected by retrospective chart review. The risk of recurrence, as determined by the ATA classification (Table II) (8) also was determined for each patient. Patients with completely excised disease without any of the following features, that is, extrathyroid extension, aggressive histopathology, vascular invasion or regional or distant metastases, are at low risk of recurrence, and patients with gross extrathyroid extension, distant metastases, or incomplete tumor resection are at high risk of recurrence. All other patients fall into intermediate-risk group for recurrence.

Patients were followed with annual ultrasound and unstimulated postoperative Tg measurements. During the time period of the study, the thyroid stimulating hormone level was maintained at less than 0.1 mIU/L in intermediate-risk patients and between 0.1 and 0.4 mIU/L in the low-risk patients. There was no difference between those who did and did not receive RAI. Recurrence was defined as any structural abnormality on examination or imaging (ultrasound or computed tomography scan) and confirmed by FNA. Variables were compared between RAI and no RAI groups for the low-risk and intermediate-risk groups using the Pearson Chi-square test. Overall survival, disease-specific survival, and recurrence-free survival (RFS) were calculated using the Kaplan-Meier method. Univariate analysis was carried out by the log rank test. Statistical analysis was carried out using JMP statistical package (SAS Institute Inc SAS Campus Drive, Cary, NC) and SPSS (IBM Company Headquarters, Chicago, IL).

Table II | ATA criteria for risk of recurrence of the disease (ref. 8)

Low risk	*Intermediate risk*	*High risk*
All of the following present:	Any of the following:	Any of the following:
No local or distant metastases	Microscopic invasion into perithyroidal soft tissues	Macroscopic tumor invasion
All macroscopic tumor resected	Cervical lymph node metastases or RAI uptake outside thyroid bed on posttreatment scan after thyroid remnant ablation	Incomplete tumor resection
No invasion of locoregional tissues	Aggressive histology or vascular invasion present	Distant metastases
No aggressive histology		Possibly thyroglobulinemia out of proportion compared with posttreatment scan
No vascular invasion		
No RAI uptake outside thyroid bed, if RAI done		

RAI, Radioactive iodine therapy.

Results

In the GAMES-classified (9) low-risk group (n = 80), 35 patients were managed with adjuvant RAI and 45 without. Comparison of patient and tumor characteristics (Table III) showed patients treated without RAI were more likely to have smaller neoplasms (82% vs 60%, P = .027). With a median follow-up time of 62 months (range, 2–116 months), there were no disease-specific deaths in either group (Table IV). There was 1 neck

recurrence in the cohort managed without RAI. The site of recurrence was the lateral neck involving levels II– IV. Patients managed without RAI had a similar RFS to patients managed with RAI (Fig 1: 96% vs 100%, P = .337). There were no factors predictive of RFS by univariate analysis (Table V).

In the GAMES-classified (9) intermediate-risk group (n = 218), 135 were managed with adjuvant RAI and 83 without. Comparison of patient and tumor characteristics (Table VI) showed patients managed without RAI were more likely to be older patients (≥45 years: 90% vs 39%, P < .0005) with smaller tumors (pT1T2: 97% vs 62%, P < .0005) and negative neck disease (N0: 56% vs 30%, P <.0005). With a median follow-up time of 59 months (range, 1–121 months), there were no disease specific deaths in either group (Table VII). There were 7 recurrences of which 6 were in the RAI cohort (5 regional, 1 distant) and 1 in the non-RAI cohort (1 regional). In the RAI group, the neck recurrences were located in the lateral neck in 4, and in the central neck in 1 patient. In the non-RAI group, the single neck recurrence was in the lateral neck. Patients managed without RAI had a similar RFS to patients managed with RAI (Fig 2: 97% vs 96%, P = .234). Factors predictive of RFS by univariate analysis (Table VIII) were pathologic T stage; RFS was 97.6% for pT1 compared with 75% for pT4 tumors, P = .011.

Table III | Patient characteristics for GAMES low-risk patients (n = 80)

Characteristic	*No. patients (%)*	*No. patients without RAI (%)*	*No. patients with RAI (%)*	*χ2 P value*
Sex				
Male	10 (13)	4 (9)	6 (17)	.268
Female	70 (87)	41 (91)	29 (83)	
Previous radiation exposure				
No	74 (93)	42 (93)	32 (91)	.748
Yes	6 (7)	3 (7)	3 (9)	
pT stage				
T1	58 (73)	37 (82)	21 (60)	.027*
T2	22 (27)	8 (18)	14 (40)	
Size of neoplasm, cm				
≤1	35 (44)	27 (60)	8 (23)	.006*
1–2	23 (29)	10 (22)	13 (37)	
2–3	16 (20)	7 (16)	9 (26)	
3–4	6 (7)	1 (2)	5 (14)	
ATA risk				
Low	76 (95)	45 (100)	31 (89)	.020*
Int	4 (5)	0 (0)	4 (11)	

**Statistically significant.*
ATA, American Thyroid Association; RAI, radioactive iodine.

Table IV | Survival event details for GAMES-classified low-risk patients

Event	*No. events (without RAI / with RAI)*	*5-y survival, %*
Overall survival	3 (3/0)	95.6
Disease-specific survival	0 (0/0)	100
Recurrence	1 (1/0)	98
Local recurrence	0 (0/0)	100
Neck recurrence	1 (1/0)	98
Distant recurrence	0 (0/0)	100

RAI, Radioactive iodine.

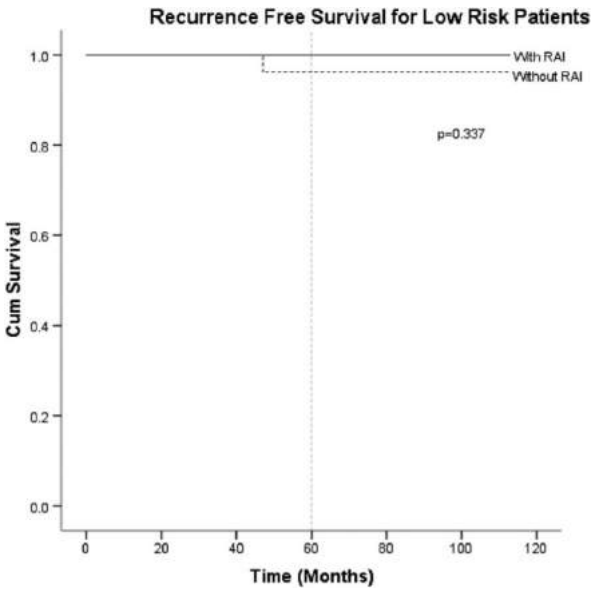


Figure 1 | RFS for low-risk patients.

Discussion

Well-differentiated PTC is the most frequent type of thyroid cancer. During the past 25 years, there has been a steep increase in incidence according to cancer registry reports from different countries (1,2). Because the majority of this increase is attributed to the discovery of small and often asymptomatic subclinical low-risk tumors (10) there has been no significant change in disease outcome (3), which has remained excellent for PTC patients. With our increased understanding of the natural history and biology of PTC, stratification systems have been developed to classify the risk of disease specific death and the risk of disease recurrence (8,11-13). The aim of these stratification systems is to better tailor the extent and intensity of treatment and follow-up. Although significant improvement has been achieved regarding the selection of the extent of thyroid surgery (lobectomy versus total thyroidectomy) based on risk features of an individual patient (14), the benefit of adjuvant RAI therapy after total thyroidectomy has not been fully clarified. This is largely due to the lack of any prospective trials on the use of RAI, with current recommendations being reliant on retrospective analyses of case series from a variety of institutions.

Postoperative RAI ablation routinely is used at some institutions to destroy residual thyroid tissue, and hypothetically, residual cancer cells and occult lymph node metastases and to decrease long-term risk of recurrent disease and disease specific mortality as well as to facilitate initial staging and subsequent follow-up (8). It is not clear, however, if this treatment paradigm benefits patients with undetectable postoperative Tg level, which implies complete clearance of all disease and all normal thyroid tissue (15,16). Tg is a glycoprotein produced only by normal or neoplastic thyroid follicular cells. After total thyroidectomy, detection of Tg signifies the presence of persistent thyroid tissue or persistent disease (17).

Some clinicians support the routine use of postoperative RAI ablation, claiming that it facilitates subsequent follow-up of patients with serial Tg measurements. However, a detectable Tg levels can occur in situations when 'residual thyroid tissue' is left behind after a subtotal or near total thyroidectomy. On the other hand, if

the surgical procedure is a true "total thyroidectomy" with no measurable Tg postoperatively, then the routine use of RAI ablation should no longer be required. In these patients, serial Tg measurements can be safely used for future follow-up and surveillance. Thus, the need for a careful and meticulous total thyroidectomy that leaves behind no residual thyroid tissue cannot be over emphasized. Current guidelines of the ATA (8) recommend administration of RAI after total thyroidectomy for DTC in patients with known distant metastases, gross extrathyroidal extension, primary tumor size >4 cm even in the absence of other greater-risk features; in select patients with tumors 1–4 cm confined to the thyroid who have lymph node metastases; and in select patients with greater-risk tumor histology that predicts moderate-to-high risk of recurrence or death from thyroid cancer. The British Thyroid Association and Royal College of Physicians in in the United Kingdom conider an even wider range of indications, recommending RAI for tumors greater than 1 cm (18). There is a general agreement that post operative RAI is not indicated in low-risk patients with PTC <1 cm confined to the thyroid (unifocal or multifocal) (8), because these patients have a very low risk of recurrence (2–8%) (ref. 19). Similarly, most studies agree on the benefit of adjuvant RAI in patients with high-risk features because RAI decreases both recurrence and death rates. (20,21). However, the impact of postoperative RAI on improvement of survival and recurrence in other low- and intermediate-risk patients is less clear (22-25).

Postoperative Tg level has been reported undetectable in up to 57% of patients after total thyroidectomy (26). indicating clearance of all disease and all normal thyroid tissue (15,16). However, postoperative Tg level has not been included among the factors impacting the decision for RAI ablation according to recent ATA guidelines (8). To evaluate the significance of an undetectable Tg after total thyroidectomy in patients with PTC, we identified patients with undetectable Tg after total thyroidectomy with/without neck dissection for PTC. Of 751 patients who had a total thyoidectomy and had a postoperative Tg measurement performed, 424 (56%) patients had an undetectable level of Tg, indicating the effectiveness of our surgical resection. For the purposes of this study we used a cut-off point of Tg level as <1 ng/mL to include patients reported in early 1999 when less-sensitive methods of Tg measurement were available (27,28).

Table V | Factors predictive of recurrence-free survival for GAMES-classified low-risk patients

	n	*5-y RFS, %*	P *value*
Sex			
Male	10	100	.712
Female	70	97.7	
Previous radiation exposure			
No	74	97.8	.739
Yes	6	100	
pT stage			
T1	58	100	.127
T2	22	93.3	
Tumor size, cm			
≤1	84	100	.315
1–2	58	100	
2–3	31	90.9	
3–4	15	100	
RAI			
No	45	96.2	.337
Yes	35	100	
ATA risk			
Low	76	97.9	.801
Int	4	100	

ATA, American Thyroid Association; RAI, radioactive iodine.

Presently we use a cut-off of unstimulated Tg <0.3 ng/mL as our definition of undetectable Tg. We stratified our patients into low-, intermediate-, and high-risk groups according to GAMES criteria for the risk of disease specific death (9) and used the postoperative Tg as a measure of completeness of resection. For the purpose of this study we only analyzed low- and intermediate-risk patients with undetectable Tg because the most controversy on the benefit of adjuvant RAI is within these 2 groups of patients.

Table VI | Patient characteristics for GAMES-classified intermediate-risk patients (*n* = 218)

Characteristic	*No. patients (%)*	*No. patients without RAI (%)*	*No. patients with RAI (%)*	*χ2 P value*
Age, y				
<45	91 (42)	8 (10)	83 (61)	<.0005*
≥45	127 (58)	75 (90)	52 (39)	
Sex				
Male	51 (23)	16 (19)	35 (26)	.260
Female	167 (77)	67 (81)	100 (74)	
Previous radiation exposure				
No	203 (93)	77 (93)	126 (93)	.873
Yes	15 (7)	6 (7)	9 (7)	
pT stage				
T1	127 (58)	71 (86)	56 (41)	<.0005*
T2	37 (17)	9 (11)	28 (21)	
T3	50 (23)	3 (3)	47 (35)	
T4	4 (2)	0 (0)	4 (3)	
pN stage				
Nx	56 (26)	31 (37)	25 (19)	<.0005*
N0	87 (40)	46 (56)	41 (30)	
N+	75 (34)	6 (7)	69 (51)	
Size of neoplasms, cm				
≤1	78 (36)	48 (58)	30 (22)	<.0005*
1–2	89 (41)	25 (30)	64 (48)	
2–3	37 (17)	7 (8)	30 (22)	
3–4	10 (5)	3 (4)	7 (5)	
>4	4 (1)	0 (0)	4 (3)	
ATA risk				
Low	125 (57)	73 (88)	52 (39)	<.0005*
Int	81 (37)	8 (10)	73 (54)	
High	12 (6)	2 (2)	10 (7)	

**Statistically significant.*

ATA, American Thyroid Association; RAI, radioactive iodine.

Table VII | Survival event details for GAMES-classified intermediate-risk patients

Event	*No events (without RAI / with RAI)*	*5-y survival, %*
Overall survival	12 (2/10)	96.2
Disease-specific survival	0 (0/0)	100
Recurrence	7 (1/6)	96.5
Local recurrence	0 (0/0)	100
Neck recurrence	6 (1/5)	97
Distant recurrence	1 (0/1)	99.5

RAI, Radioactive iodine.

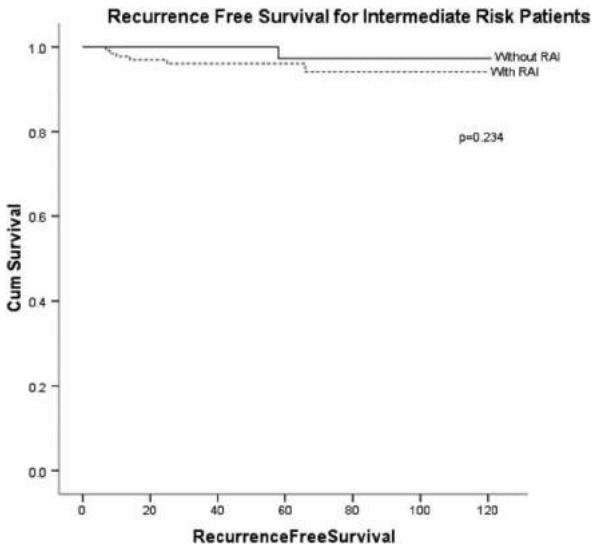


Figure 2 | RFS for intermediate-risk patients.

Our data on the GAMES-classified (9) low-risk group of patients showed that we selected patients with tumors <2 cm not to receive adjuvant RAI. When we used the ATA risk of recurrence stratification (8), all these patients were categorized as low risk for recurrence. RFS of these patients was equivalent to the patients who received RAI thus validating our selection criteria. We can therefore conclude that patients classified as low risk for death using GAMES criteria and low risk for recurrence using ATA guidelines can be safely managed without RAI after total thyroidectomy if they have an undetectable postoperative Tg.

Our data on GAMES-classfied (9) intermediate-risk group patients showed that the patients who did not receive RAI were mainly older patients (≥45 years) with small neoplasms (pT1T2) and with a pathologically negative neck. In this group of patients we reserve adjuvant RAI for mainly young patients (<45 years) with large tumors (pT3T4) and positive neck disease. The RFS for the RAI and non RAI groups was equivalent, again validating our treatment policy. Analysis of the intermediate-risk patients into low-, intermediate-, and high-groups according to the ATA risk of recurrence stratification (8) revealed that the majority of patients who we selected not to receive adjuvant RAI were classified as low risk for recurrence. We can therefore conclude that patients classified as intermediate risk for death using GAMES criteria and low risk for recurrence using ATA guidelines can be safely managed without RAI following total thyroidectomy and an un-detectable postoperative Tg.

There are some limitations of our data due to the retrospective nature of the study design. Such retrospective analysis can never fully take into account all the selection bias (institutional, physician, and patient related) that is inherent in these studies. However, unlike national cancer databases, all our patients were treated within a single tertiary referral cancer center with an internal consistency in approach to surgery, histopathologic reporting, postoperative treatment and follow-up. In addition, all cases were reviewed for treatment and outcome data by a multidisciplinary team with a high level of clinical experience in the treatment of thyroid cancer. Our data strongly support stringent selection criteria for postoperative use of RAI in patients with undetectable Tg level after total thyroidectomy. Our data suggest that properly selected low-risk (GAMES) patients with undetectable levels of Tg (patients <45 yrs with pT1 tumors) and properly selected intermediate

risk (GAMES) patients with undetectable Tg (patients >45 yrs with small T1T2 tumors and negative necks) can be safely managed without RAI.

Table VIII | Factors predictive of recurrence-free survival for GAMES-classified intermediate-risk patients

	n	*5-y RFS, %*	P *value*
Age, y			
<45	91	95.3	.117
≥45	127	97.5	
Sex			
Male	51	97.8	.638
Female	167	96.1	
Previous radiation expsure			
No	203	96.2	.458
Yes	15	100.0	
pT stage			
T1	127	97.6	.011*
T2	37	97.1	
T3	50	95.6	
T4	4	75.0	
pN stage			
N0	87	97.3	.090
N+	75	94.4	
Tumor size, cm			
≤1	78	97.4	.350
1–2	89	96.3	
2–3	37	94.4	
3–4	10	100.0	
>4	4	100.0	
RAI			
No	83	97.2	.234
Yes	135	96.1	
ATA risk			
Low	125	97.5	.231
Int	81	96.0	
High	12	90.9	

**Statistically significant.*
ATA, American Thyroid Association; RAI, radioactive iodine.

Because recurrence rates are so low with un-stimulated Tg measurements, one can argue that the routine use of stimulated Tg in these patients to detect biochemical disease without any anatomic demonstration has little practical value in clinical management. It is important to state that our data cannot conclude that intermediate-risk patients who are young with large tumors and positive neck disease do not benefit from RAI. Indeed it can be argued that the use of RAI in the patients with T3T4 tumors prevented local recurrence and the use of RAI in the patients with positive neck disease reduced the incidence of neck recurrence.

The selective use of RAI in patients with thyroid cancer has other important implications, such as the reduction in the potential risk of second primary cancers and the avoidance of RAI-related side effects. Salivary glands changes are probably among the most frequent adverse effects after single and moderate dose of RAI treatment. They are usually transitory (occurring in up to 39% of patients), but can be permanent in up to 5%, (6,7) resulting in chronic dry mouth and alteration of taste. Recent data also have indicated that the incidence of a second malignancy after radioiodine might be higher than previously thought (4,5) with a rising incidence even in patients with low risk tumors after RAI therapy (29). Although these adverse effects are infrequent,

their implication is important because the benefit of RAI in reducing disease specific mortality and recurrence rates in PTC without distant metastases has not been definitely proven, especially in lower-risk groups.

In conclusion, we can say that a methodical total thyroidectomy removing all gross thyroid tissue, particularly at the superior pole, Berry's ligament, and the pyramidal lobe, as well as removing all clinically suspicious lymph nodes at the time of thyroidectomy, can result in an undetectable postoperative Tg level. Properly selected low- and intermediate-risk group patients can then be safely managed without RAI with no increase in recurrence. In general, our data suggest that in PTC patients with an undetectable levels of Tg after total thyroidectomy, the majority of patients (both <45 years and >45 years of age) with pT1T2 tumors and no other adverse pathologic features can be safely managed without RAI. Because the majority of newly diagnosed PTC are pT1, the philosophy of selective postoperative RAI management can dramatically reduce the number of patients receiving RAI and therefore avoid the unwanted side effects of such treatment as well as reduce the risk of second primary cancers.

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Chapter 6

Undetectable Thyroglobulin Levels in Poorly Differentiated Thyroid Carcinoma Patients Free of Macroscopic Disease After Initial Treatment: Are They Useful?

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Ann Surg Oncol 2015; 22(13):4193–4197

Abstract

Background

Predictive role of undetectable thyroglobulin (Tg) in patients with poorly differentiated thyroid carcinoma (PDTC) is unclear. Our goal was to report on Tg levels following total thyroidectomy and adjuvant RAI in PDTC patients and to correlate Tg levels with recurrence.

Methods

Forty patients with PDTC with no distant metastases at presentation (M0) and managed by total thyroidectomy and adjuvant RAI were identified from a database of 91 PDTC patients. Of these, 31 patients had Tg values recorded and formed the basis of our analysis. A nonstimulated Tg level <1 ng/ml was used as a cutoff point for undetectable Tg levels. Association of patient and tumor characteristics with Tg levels was examined by Chi-square test. Recurrence-free survival (RFS) stratified by postop Tg level was calculated by Kaplan-Meier method and compared by log-rank test.

Results

Twenty patients had undetectable Tg (<1 ng/ml) and 11 had detectable Tg (≥1 ng/ml; range 2-129 ng/ml) following surgery. After adjuvant RAI, 24 patients had undetectable Tg (<1 ng/ml) and 7 had detectable Tg (≥1 ng/ml; range 1-57 ng/ml). Patients with undetectable Tg were less likely to have pathologically positive margins compared to those with detectable Tg (33 vs. 72 % respectively; p = 0.03). Patients with undetectable Tg levels had better 5-year regional control and distant control than patients with detectable Tg level (5-year regional recurrence-free survival 96 vs. 69 %; p = 0.03; 5-year distant recurrence-free survival 96 vs. 46 %, p = 0.11).

Conclusion

Postoperative thyroglobulin levels in subset of patients with PDTC appear to have predictive value for recurrence. Patients with undetectable Tg have a low rate of recurrence.

Introduction

Thyroglobulin (Tg) is a specific product of thyroid follicular cells. Serum Tg levels have been widely used as a postoperative marker for residual or recurrent tumor in differentiated thyroid carcinoma (DTC). Patients with low-risk DTC with undetectable postoperative Tg values have very low risk of recurrence (1-3). On the other hand, studies on Tg values in poorly differentiated thyroid carcinoma (PDTC) have been limited and the predictive role of an undetectable Tg in PDTC is unknown (4,5). Therefore, the purposes of our study were to report on Tg values in PDTC following total thyroidectomy and adjuvant RAI, correlate Tg values with outcome and determine if undetectable Tg predicts for low risk of recurrence in PDTC.

Materials and Methods

Following Institutional Review Board approval, we performed a retrospective review of our thyroid cancer database for patients with PDTC, treated with primary surgery, with or without adjuvant RAI at MSKCC from 1986 to 2009. 91 patients with primary PDTC were identified. Diagnosis was confirmed by two independent pathologists (R.A.G. and D.L.C.) and was based on histological and/or immunohistochemical evidence of follicular cell differentiation with presence of tumor necrosis and/or ≥5 mitoses per 10 high-power fields (400x) (6). Of 91 PDTC patients, 67 were M0 (had no distant metastasis at presentation). Of 67 M0 patients, 56 had total thyroidectomy, of whom 43 also received adjuvant RAI. After exclusion of 12 patients (3 due to external radiotherapy and 9 due to unknown Tg values), data on Tg values were available in 31 patients. These 31 PDTC patients formed the basis of our analysis. All cases were considered to express thyroglobulin based upon the histologic identification of colloid on hematoxylin and eosin section and/or the presence of positive thyroglobulin on immunohistochemistry (12 of the cases were stained for thyroglobulin and all were positive for this protein by immunostaining).

Undetectable serum Tg was defined as an unstimulated Tg < 1 ng/ml measured at least 5 weeks after total thyroidectomy and at least 3 months (3-11 months; median 6 months) after adjuvant RAI. 26 PDTC patients in our cohort were treated from 2000 to 2009, whereas five patients were treated from 1992 to 1998. Sensitivity of Tg assay ranged from <0.4 to 0.6 ng/ml for the period 2000-2009 and <0.9-1 ng/ml during 1992-1998. Therefore a cutoff point of <1 ng/ml was used to define an undetectable Tg levels. By choosing this level, we are able to encompass all patients from both time periods into our definition. Tg antibodies were routinely performed in conjunction with the Tg assay and samples with Tg antibody interference were excluded from our analysis. In the patient follow-up, TSH levels were maintained at <0.1-0.5 mIU/L. Patient charts were reviewed for patient characteristics, clinical presentation, tumor pathological features, treatment, recurrences, and survival. Staging was classified according to the 7th edition of AJCC Cancer Staging Manual (7). All 31 patients had total thyroidectomy with complete gross tumor removal (29 had total thyroidectomy and 2 had extended total thyroidectomy with removal of all tissues involved by extrathyroidal spread). Select patients also underwent neck dissection for clinically suspicious disease at the time of total thyroidectomy (determined by neck palpation, cross sectional imaging or ultrasound). In our institution, we do not routinely perform elective dissection of either the central compartment or lateral compartment lymph nodes. If clinically suspicious lymph nodes are present in the lateral compartment, dissection of neck levels II-V is performed. For clinically suspicious nodes in the central compartment, bilateral paratracheal lymph node dissections are performed. If small, 1-2 lymph nodes are present in the central compartment, lymph node sampling is performed. In addition to surgical removal of all gross tumor, patients received adjuvant RAI (30-391 mCi; median dose 150 mCi). Only three patients received doses under 100 mCi, i.e., 29.5, 78.4, and 97.7 mCi. Therefore, the overwhelming majority of our patients received therapeutic RAI dose >100 mCi. All patients had diagnostic

scan before adjuvant RAI and adjuvant RAI dose was determined based on the diagnostic scan and stage of PDTC.

The association of patient and tumor characteristics with Tg levels (obtained postadjuvant RAI) was examined by using the Pearson Chi-square test. Overall survival (OS), disease-specific survival (DSS), recurrence-free survival (RFS), regional recurrence-free survival (RRFS), and distant recurrence-free survival (DRFS) were calculated by the Kaplan-Meier method, stratified by Tg levels (obtained post adjuvant RAI) and compared using the log-rank test. Recurrence was defined as a new local, regional, or distant finding, in a patient clinically free of disease for at least 6 months following initial therapy, that was proven by biopsy or identified on computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), or RAI scanning. Death from disease was defined if it was confirmed by death certificate or hospital summary. A level of significance alpha value $p < 0.05$ was used to determine significance. Statistical analysis was performed by SPSS (IBM Company Head-quarters, 233 S. Wacker Drive, 11th Floor, Chicago, IL 60606).

Results

Tg Values in PDTC Patients and Phenotype of PDTC

Following surgery, 20 patients had undetectable Tg (<1 ng/ml) and 11 had detectable Tg (≥ 1 ng/ml; range 2-129 ng/ml). After adjuvant RAI, 24 patients had undetectable Tg (<1 ng/ml) and 7 had detectable Tg (≥ 1 ng/ml; range 1-57 ng/ml). The phenotypes of the 24 patients with undetectable Tg following adjuvant RAI were papillary (n = 15), tall cell variant (n = 5), and mixed phenotype (n = 4). The phenotypes of the seven PDTC patients with detectable Tg were papillary (n = 3), tall cell variant (n = 1), follicular (n = 1), and mixed phenotype (n = 2). Four patients that converted from detectable Tg levels to undetectable Tg levels following adjuvant RAI had papillary phenotype (three patients) and mixed phenotype (papillary, follicular, and Huer thle cell carcinoma; one patient). Three of four patients were older males (≥ 45 years), whereas one patient was a young female (29 years old). Three of four patients had smaller tumors (≤ 4 cm), whereas one patient had a larger tumor (>4 cm). Only one patient had extrathyroidal extension (microscopic). Respective staging of the PDTC that converted from detectable Tg levels to undetectable Tg levels were: T1NxM0, T2NxM0, T3NxM0, T3N1bM0.

Association of Tg Levels with Patient and Tumor Characteristics of PDTC

Patients with undetectable Tg (<1 ng/ml) and detectable Tg levels (≥ 1 ng/ml) post adjuvant RAI showed no significant age or gender differences (Table 1). Patients in both groups were predominantly older (≥45 years): 62 % of patients with undetectable Tg and 71 % of patients with detectable Tg ($p = 0.664$). Patients who achieved an undetectable Tg postadjuvant RAI were predominantly females (71 %) compared with patients with detectable Tg (43 %; $p = 0.173$).

When pathological tumor characteristics were examined, only pathologically positive margins were significantly associated with detectable Tg compared with undetectable Tg (72 vs. 33 % respectively; $p = 0.03$). Patients with undetectable Tg had less advanced pathological tumor characteristics compared with patients with detectable Tg. Patients with undetectable Tg were less likely to have large primary tumors (>4 cm; 29 vs. 43 %; $p = 0.495$), higher pT stage (pT3/T4; 67 vs. 100 %; $p = 0.076$), extrathyroid extension (50 vs. 86 %; $p = 0.092$), and pathologically positive neck nodes (33 vs. 71 %; $p = 0.072$), respectively.

Table 1 |Association of Tg levels with patient and tumor characteristics

Variable	Tg < 1 ng/ml No. (%)	Tg ≥ 1 ng/ml No. (%)	χ^2, *p* value
Age (years)			
<45	9 (38 %)	2 (29 %)	0.664
≥45	15 (62 %)	5 (71 %)	
Sex			
M	7 (29 %)	4 (57 %)	0.173
F	17 (71 %)	3 (43 %)	
pT size (cm)			
≤4	17 (71 %)	4 (57 %)	0.495
>4	7 (29 %)	3 (43 %)	
pT stage			
T1/T2	8 (33 %)	0 (0 %)	0.076
T3/T4	16 (67 %)	7 (100 %)	
ETE			
No	12 (50 %)	1 (14 %)	0.092
Yes	12 (50 %)	6 (86 %)	
Minimal	7 (54 %)	0 (0 %)	
Gross	6 (46 %)	7 (100 %)	
Margins			
Negative	15 (63 %)	1 (14 %)	**0.033**
Positive/close	8 (33 %)	5 (72 %)	
Unknown	1 (4 %)	1 (14 %)	
pN stage			
pNx/pN0	16 (67 %)	2 (29 %)	0.072
Nx	10 (63 %)	0 (0 %)	
N0	6 (37 %)	2 (100 %)	
pN+	8 (33 %)	5 (71 %)	
N1a	5 (63 %)	1 (20 %)	
N1b	3 (37 %)	3 (60 %)	
N1x	0 (0 %)	1 (20 %)	

Bold value indicates statistically significant

ETE extrathyroid extension, pNx clinically negative neck

Outcome in PDTC Stratified by Tg Levels

Of 31 PDTC patients in our cohort, 6 patients (19 %) died of whom 4 died of the disease. There were 6 recurrences: 1 regional and 5 distant and regional. With a median follow-up of 49 months (17–143 months), there was no significant difference in 5-year OS or DSS between patients with detectable and undetectable Tg (5-year OS: 85.7 vs. 86.3 %; p = 0.38; 5-year DSS: 100 vs. 95.8 %; p = 0.45). Patients with an undetectable Tg had significantly better 5-year recurrence control (RFS) compared with patients with detectable Tg (96 vs. 26 %; p = 0.001; Fig. 1). Patients with undetectable Tg levels had significantly better 5-year regional control (RRFS: 96 vs. 69 %; p = 0.03; Fig. 2); of 6 patients with regional recurrence during 5-year follow-up, 2 (33 %) had undetectable Tg. Patients with undetectable Tg levels also had better 5-year distant control (DRFS: 96 vs. 46 % vs. p = 0.11; Fig. 3). Of 5 patients with distant recurrence during 5 year follow-up, 2 (40 %) had undetectable Tg. Two of 24 patients with undetectable Tg had distant recurrences: 1 to lung and 1 to lung and bone. Three of seven patients with detectable Tg had distant recurrences: 1 to lung, 1 to lung and bones, and 1 to lung, bones, and liver. Two patients with undetectable Tg that developed both distant and regional recurrence received EBRT (external beam radiation therapy) and EBRT+ RAI, respectively. Three patients with detectable Tg that developed both regional and distant recurrences received: (1) neck dissection and RAI; (2) neck dissection and

EBRT/experimental systemic therapy; and (3) RAI plus an operation of solitary lung metastasis, respectively. One patient with detectable Tg levels that developed regional recurrence received a neck dissection.

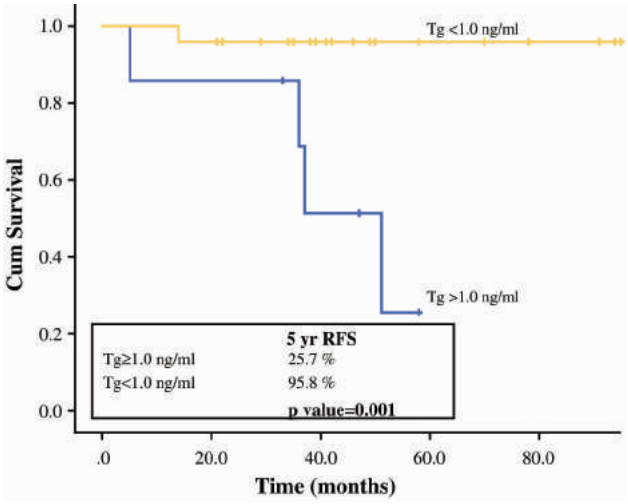

Figure 1 | Five-year, recurrence-free survival (RFS) stratified by Tg level

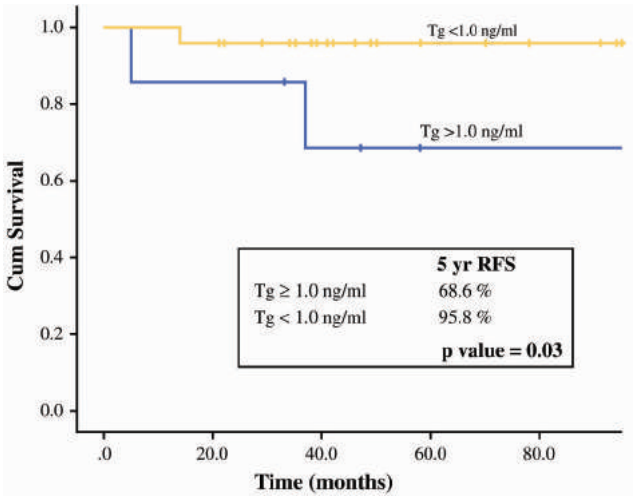

Figure 2 | Five-year, regional, recurrence-free survival (RFS) stratified by Tg level

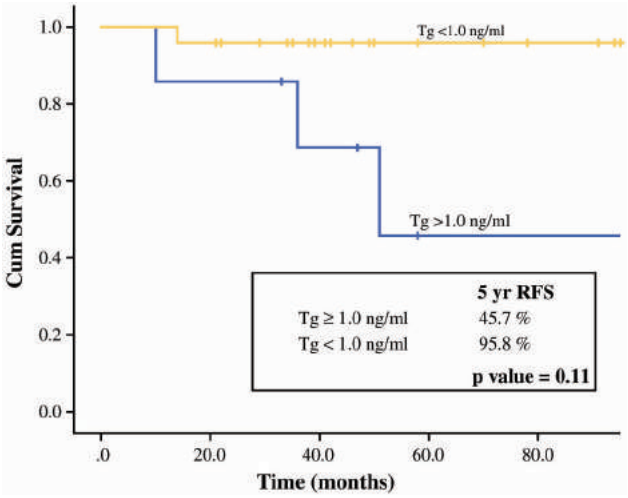

Figure 3 | Five-year, distant, recurrence-free survival (RFS) stratified by Tg level

Discussion

Poorly differentiated thyroid carcinoma is a rare type of thyroid cancer with biologically and histologically intermediate characteristics on a progression scale from DTC to undifferentiated or anaplastic thyroid carcinoma (AC) (8,9). Despite loss of some of the well-differentiated features, PDTC still contains colloid and produces Tg (10,11). This is due to tumor heterogeneity within the primary tumor mass. However, due to the rarity of PDTC, data on Tg production and the possible predictive role in PDTC are unclear (4,5).The objective of our study was to report on Tg values in PDTC following total thyroidectomy and adjuvant RAI, correlate Tg values with outcome in PDTC, and determine if an undetectable Tg level predicted low-risk for recurrence.

Based on the observations from our study, thyroglobulin appears to have a prognostic role in the prediction of recurrence in patients with PDTC. In our study, we found that PDTC patients who were free of macroscopic disease after initial treatment and had undetectable Tg showed significantly better 5-year RFS compared with patients with detectable Tg (96 vs. 26 %; p = 0.001). In particular, 5-year regional control and distant control was better for our patients with undetectable Tg levels (5-year RRFS 96 vs. 69 %; p = 0.03; 5-year DRFS 96 vs. 46 % vs. p = 0.11). Similar to our study of PDTC, the study by van Dijk et al. of DTC found that after initial surgery and adjuvant RAI therapy, patients with detectable Tg and negative posttherapeutic WBS had significant earlier and more recurrences than patients without detectable Tg (12). Survival in both DTC groups with detectable and undetectable Tg was comparable as we found in our PDTC cohort.

As in DTC, we also report that patients who had detectable Tg were more likely to have larger tumors, ETE, positive neck disease, and also positive margins following thyroidectomy. These observations mirror those found in differentiated thyroid cancer.

Our data suggest that measurement of serum Tg is valuable in the follow-up of both DTC and PDTC. If an undetectable Tg level is obtained after initial surgery and adjuvant RAI, then the risk of recurrence is low. However, this is not absolute, because two of our patients with undetectable Tg levels had recurrence (2/24). This is most likely due to tumor heterogeneity and the presence of a less differentiated tumor component.

Therefore, if undetectable Tg level is obtained after initial treatment, the physician must still be aware that a small subset of PDTC patients might recur due to the presence of a less differentiated tumor component.

Our study is not without its limitations however. The study is retrospective and therefore susceptible to the deficiencies associated with retrospective data collection. In addition, our sample size was small (31 patients). However, PDTC is a rare type of thyroid cancer and our cohort was created from one of the largest available series of PDTC patients (91 patients) who have been treated at a single tertiary care center. It is unlikely that other centers will have any more detailed information on the role of Tg in PDTC. Another strength of the cohort was that all had pathology review and that a single diagnostic criteria based on high grade features (mitosis and necrosis) was used to classify the patients. These diagnostic criteria have been shown to define a biologically more homogenous group of tumors (6). Selection bias is another clear limitation to the data. The 5-year OS for our cohort (86 %) was better than most reports on 5-year OS in PDTC, which range from 62 to 85 %.13–16 This reflects the fact that our 31 PDTC patients represent a cohort that was free of distant metastases at presentation (M0) and free of macroscopic disease after initial treatment with surgery and adjuvant RAI. In addition, it is possible that the selected cohort of tumors has heterogeneity with areas of differentiated carcinoma coexisting with PDTC that still secrete Tg. Lastly, although we stated that there was no difference in OS and DSS between patients with detectable and undetectable Tg levels, the follow-up of our cohort was limited to 49 months. Even with PDTC, recurrences and deaths can occur after our median follow-up time of 49 months.

Despite these limitations, we conclude that many PDTC, who are M0 at presentation, retain the ability to produce Tg after initial treatment with surgery and adjuvant RAI. Those PDTC patients with undetectable Tg levels show lower rates of distant recurrence and significantly lower rates of regional recurrence. Tg therefore may serve as a predictor for recurrence in PDTC. However, in a small subset of PDTC patients with undetectable Tg after initial treatment, undetectable levels may not be a reliable indicator for recurrence due to presence of a more aggressive and less differentiated component in a heterogenous tumor. We emphasize the need for future studies with a larger sample size to reach a definitive conclusion. Further studies, most likely with multi-institutional collaboration due to the rarity of these tumors, may help to confirm our observations.

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Part III

Genomic Characteristics of Poorly Differentiated Thyroid Cancer

Chapter 7

Frequent Somatic TERT Promoter Mutations in Thyroid Cancer: Higher Prevalence in Advanced Forms of the Disease

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J Clin Endocrinol Metab 2013; 98(9): E1562–E1566

Abstract

Background

TERT encodes the reverse transcriptase component of telomerase, which adds telomere repeats to chromosome ends, thus enabling cell replication. Telomerase activity is required for cell immortalization. Somatic *TERT* promoter mutations modifying key transcriptional response elements were recently reported in several cancers, such as melanomas and gliomas.

Objectives

The objectives of the study were: 1) to determine the prevalence of *TERT* promoter mutations C228T and C250T in different thyroid cancer histological types and cell lines; and 2) to establish the possible association of *TERT* mutations with mutations of *BRAF*, *RAS*, or *RET/PTC*.

Methods

TERT promoter was PCR-amplified and sequenced in 42 thyroid cancer cell lines and 183 tumors: 80 papillary thyroid cancers (PTCs), 58 poorly differentiated thyroid cancers (PDTCs), 20 anaplastic thyroid cancers (ATCs), and 25 Hurthle cell cancers (HCCs).

Results

TERT promoter mutations were found in 98 of 225 (44%) specimens. *TERT* promoters *C228T* and *C250T* were mutually exclusive. Mutations were present in 18 of 80 PTCs (22.5%), in 40 of 78 (51%) advanced thyroid cancers (ATC + PDTC) ($P = 3 \times 10^{-4}$ vs PTC), and in widely invasive HCCs (4 of 17), but not in minimally invasive HCCs (0 of 8). *TERT* promoter mutations were seen more frequently in advanced cancers with *BRAF/RAS* mutations compared to those that were *BRAF/ RAS* wild-type (ATC + PDTC, 67.3 vs 24.1%; $P < 1 \times 10^{-4}$), whereas *BRAF*-mutant PTCs were less likely to have *TERT* promoter mutations than *BRAF* wild-type tumors (11.8 vs 50.0%; $P = .04$).

Conclusions

TERT promoter mutations are highly prevalent in advanced thyroid cancers, particularly those harboring *BRAF* or *RAS* mutations, whereas PTCs with *BRAF* or *RAS* mutations are most often *TERT* promoter wild type. Acquisition of a *TERT* promoter mutation could extend survival of *BRAF*- or *RAS*-driven clones and enable accumulation of additional genetic defects leading to disease progression.

Introduction

Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is usually absent in nonimmortalized cells but is expressed at functionally significant levels in the vast majority of human cancer cells, enabling their replicative immortality (1). The *TERT* gene encodes the reverse transcriptase component of the telomerase complex, and its overexpression in mouse models, such as in K5-Tert transgenic mice, leads to an increased incidence of cancer (2, 3). High telomerase activity and *TERT* expression have been reported in thyroid tumors, particularly in the advanced forms of the disease, but are absent in normal thyroid tissues (4–6).

Mutations in the proximal promoter of *TERT* have been identified recently as a highly frequent event in melanoma, particularly in the metastatic forms of the disease. The two recurrent, nonoverlapping somatic mutations identified (chr5:1,295,228C>T and chr5:1,295,250C>T, hereafter named C228T and C250T, respectively) conferred a 2- to 4-fold increase in *TERT* transcriptional activity, presumably through the generation of novel consensus binding sites in the *TERT* promoter for E twenty-six (ETS) transcription factors (7, 8).

Although *TERT* C>T transitions could be enhanced by exposure to UV light, these mutations were not restricted to melanoma and were present in 24 of 150 (16%) cell lines from the Cancer Cell Line Encyclopedia. Killela et al (9) extended the original study by surveying 1230 tumors of 60 different types and concluded that *TERT* promoter mutations occurred frequently (>15%) in a subset of 9 tumor types derived from cells with low rates of selfrenewal, including gliomas, liposarcomas, and hepatocellular carcinomas. These reports prompted us to evaluate the extent and characteristics of TERT promoter mutations in follicular cell-derived thyroid cancer specimens.

Materials and Methods

Patient tissue samples

Our series comprised 183 thyroid tumors obtained from sur-gical pathological specimens and included 80 papillary thyroid cancers (PTCs; 29 from Memorial Sloan-Kettering Cancer Center [MSKCC], New York, and 51 from Nagasaki University, Japan), 58 poorly differentiated thyroid cancers (PDTCs), 20 anaplastic thyroid cancers (ATCs), and 25 Hurthle cell cancers (HCCs). In addition, we screened 42 human thyroid cancer cell lines. The MSKCC cases of PTC, PDTC, HCC, and ATC were randomly selected from the pathology department files of the institution. The thyroid carcinomas were classified according to the last World Health Organization classification of endocrine tumors, except for PDTC (10). The latter tumor was defined as a carcinoma displaying high mitotic activity (≥ 5 mitosis/10 high-power fields, x400), and/or tumor necrosis, and showing follicular cell differentiation at the morphological or immunohistochemical level (11). The study was approved by the Institutional Review Board of MSKCC. Informed consent was also obtained for all Japanese samples.

TERT mutation testing

The TERT proximal promoter was amplified from sample DNA by a nested PCR approach, using primers and conditions previously described (8), and was subsequently sequenced on an ABI3730 capillary sequencer (Applied Biosystems). Genotyping for *BRAF* (all known point mutations) or *RAS* mutations (codons 12, 13, and 61 for all 3 *RAS* genes) was performed by either mass spectometry or Sanger sequencing as previously described (12). *RET/PTC* rearrangements were detected on tumor cDNA as previously reported (12). Statistical

differences in mutation distributions were assessed by a two-sided Fisher's exact test using GraphPad Prism software version 5.04 (GraphPad Software, Inc).

Results

TERT promoter mutations were present in 44% (98 of 225) of the thyroid cancer specimens (Table 1). *TERT* C228T was more common (67 of 225) than C250T (31 of 225), and the two did not overlap (Figure 1).

TERT mutations showed a significantly uneven distribution between tumors of different histological grades (Table 1). They were comparatively infrequent in well-differentiated PTCs (18 of 80, or 22.5%). In contrast, advanced thyroid tumors (ie, ATC and PDTC) were almost twice as likely to harbor *TERT* mutations (40 of 78, or 51.3%; P= .0003). Regarding the less frequent HCC group, only widely invasive tumors harbored *TERT* mutations (4 of 17, or 23.5%), whereas none of their minimally invasive counterparts had these defects (0 of 8). Cell lines showed the highest rate of *TERT* mutations (37 of 42, or 88.1%), suggesting that this may be a common requirement for immortalization in cell culture (Supple-mental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Table 1 | *TERT* Promoter Mutations in Thyroid Cancer Tumors and Cell Lines

		TERT Promoter Mutations, n (%)				
Group	**n**	**Wild-Type**	**C228T**	**C250T**	**C228T or C250T**	***P* Value[a]**
PTC (MSKCC)	29	21 (72.4)	5 (17.2)	3 (10.3)	8 (27.6)	
PTC (Japan)	51	41 (80.4)	5 (9.8)	5 (9.8)	10 (19.6)	
PTC (all)	80	62 (77.5)	10 (12.5)	8 (10.0)	18 (22.5)	
PDTC	58	28 (48.3)	18 (31.0)	12 (20.7)	30 (51.7)	.0005
ATC	20	10 (50.0)	10 (50.0)	0 (0.0)	10 (50.0)	.0241
Advanced thyroid cancers (PDTC + ATC)	78	38 (48.7)	28 (35.9)	12 (15.4)	40 (51.3)	.0003
HCC, minimally invasive (HCC-MIN)	8	8 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
HCC, widely invasive (HCC-WIDE)	17	13 (76.5)	3 (17.6)	1 (5.9)	4 (23.5)	.2689
Thyroid cancer cell lines	42	6 (14.3)	26 (61.9)	10 (23.8)	36 (85.7)	<.0001

[a] P values were derived from Fisher's exact test, using "PTC (all)" as the reference group, with the exception of HCC, where "HCC-MIN" was used.

We also evaluated the co-occurrence of *TERT* promoter mutations with alterations in known thyroid cancer driver genes, such as *BRAF*, *RAS*, and *RET/PTC* (Figure 1 and Supplemental Table 2). We observed a significant co-occurrence of *TERT* mutations with advanced thyroid tumors harboring *BRAF* or *RAS* alterations compared to those that were wild-type for these genes (n = 78; ATC + PDTC, 67.3 vs 24.1%; P = .0004; Figure 1). Accordingly, most of the advanced tumors without mutations in known drivers clustered in the *TERT* wild-type group (17 of 24). By contrast, we found a reciprocal association in the subset of PTCs that were genotyped for driver gene alterations. Thus, tumors were less likely to have *TERT* promoter mutations if they harbored a mutation in *BRAF* or *RAS* (n = 29; 11.8 vs 50.0%; P = .04; see Figure 1). *TERT* promoter mutations generate de novo consensus binding sites for the ETS family of transcription factors. Because these are components of the MAPK transcriptional output, these data suggest that a MAPK-independent alteration might be driving the transformation of *TERT* promoter wild-type PDTCs and ATCs.

Finally, mutation at *TERT* c.-57A>C, described in the germline of a family with cutaneous melanoma (8), was not found in any of the thyroid tumors assessed for that specific locus (0 of 82).

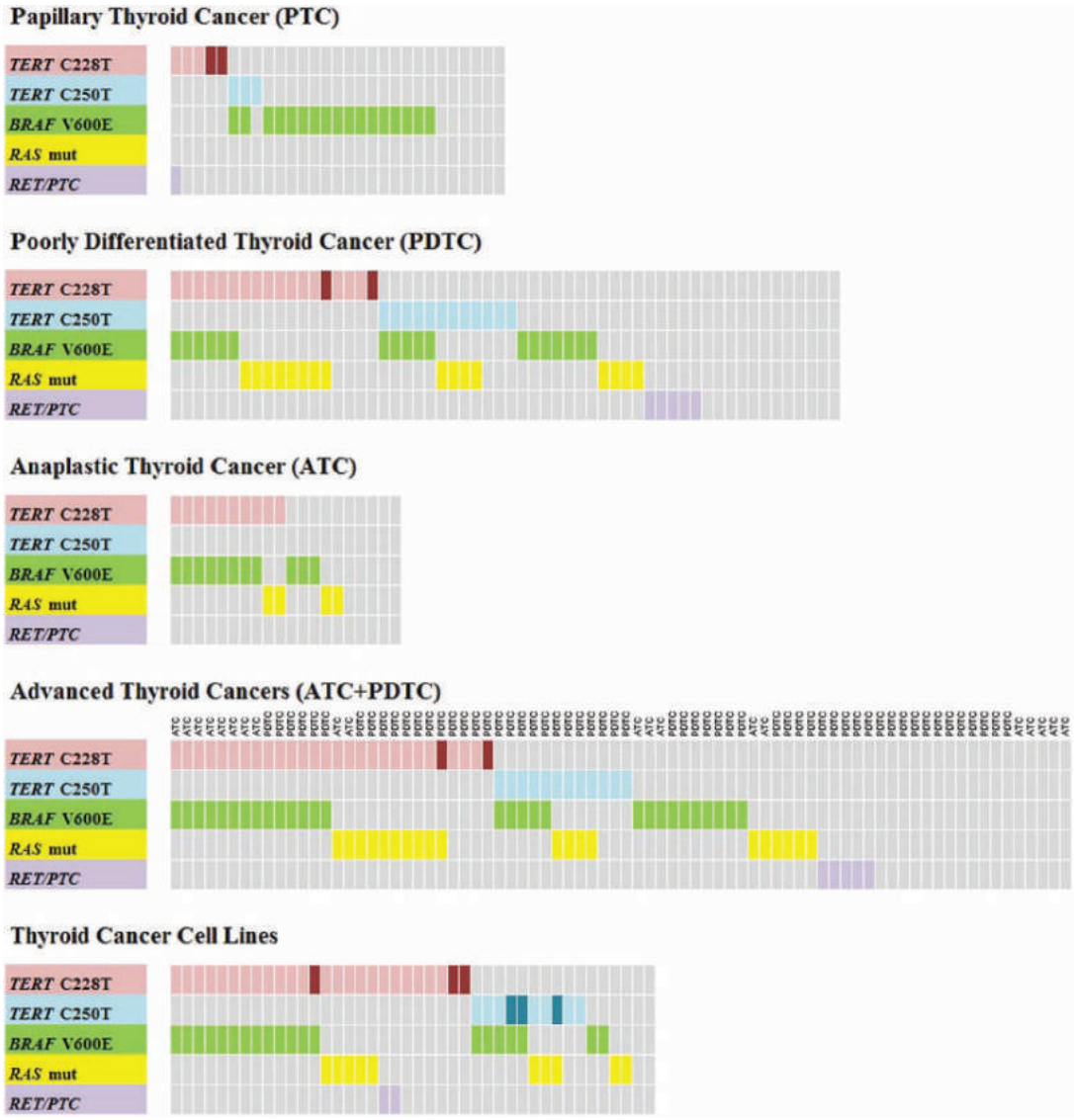

Figure 1 | Concordance of *TERT* promoter and known thyroid driver genes (*BRAF*, *RAS*, *RET/PTC*) in thyroid cancer tumors and cell lines. Each cell represents one sample. Colored and gray shadings denote mutant and wild-type status, respectively. For *TERT* mutations, darker shading represents homozygous mutations. Detailed numbers can be found on Supplemental Table 2.

Discussion

This is the first study reporting a high frequency of *TERT* promoter mutations in follicular cell-derived thyroid carcinomas. We found an overrepresentation of *TERT* promoter mutations in advanced thyroid cancers, as well as a significant co-occurrence with mutations in *BRAF* and *RAS* in this subset of tumors. *TERT* C228T and C250T mutations appeared in a strict nonoverlapping fashion, suggesting that either is sufficient to drive the phenotype.

Mutations in the promoter of *TERT* were recently identified as common events in melanomas, glioblastomas, bladder carcinomas, and other tumors (7–9, 13). *TERT* mutations are enriched in advanced cancers, such as metastatic melanomas (8) and adult primary glioblastomas (9) with respect to their less aggressive counterparts. Our results show that this is also the case in thyroid cancers, where *TERT* mutations were more prevalent in advanced forms of the disease (51%) compared to well-differentiated tumors (22%). Hence, *TERT* promoter mutations may be biomarkers of tumor progression. Deep-sequencing methods would be more rigorous screening approaches to identify *TERT* mutations in advanced disease, particularly for ATC tumors with heavy macrophage infiltration (14, 15) because it is likely that Sanger sequencing underrepresented the *TERT* mutation prevalence in these cancers.

Killela et al (9) proposed that *TERT* promoter mutations may be more common in cancers derived from terminally differentiated cells, which have a low self-renewing capacity, whereas tissues that are rapidly renewing have alternative mechanisms to maintain telomerase lengthening, and thus would be less likely to benefit from activating mutations in *TERT*. Thyroid cells have a very low mitotic rate postnatally (16). Hence, the high rate of mutations observed in thyroid cancer is consistent with this hypothesis.

We found a significant overrepresentation of *TERT* promoter mutations in thyroid tumors harboring alterations in *BRAF* or *RAS* genes. A likely functional consequence of both C228T and C250T is to create de novo consensus binding sites for ETS factors in the *TERT* promoter (7, 8). MAPK activation, through either *BRAF* or *RAS* mutations, induces expression of members of the ETS transcription factor family (17). Conceivably, acquisition of a *TERT* promoter mutation could extend the lifespan of *BRAF*- or *RAS*-driven clones and enable accumulation of additional genetic defects leading to the development of more advanced forms of the disease. This may help explain the paradoxical finding that well-differentiated PTCs harboring *BRAF* or *RAS* mutations are less likely to harbor *TERT* promoter mutations than PTCs that are wild-type for these oncogenes, whereas BRAF- or *RAS*-mutant PDTCs and ATCs are markedly enriched for these *TERT* defects. These data raise the possibility that *TERT* promoter mutations may be relevant prognostic markers in thyroid cancer and should help refine the molecular taxonomy of the disease. It should be noted that mutations in the *TERT* promoter, although remarkably frequent, may be only 1 of the potential mechanisms of illegitimate activation of *TERT*, which may include aberrant methylation of the *TERT* promoter (18) or inactivating mutations in the *ATRX* gene, a Rad54-like ATP-driven DNA translocase, the loss of function of which leads to telomere lengthening (19).

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Chapter 8

Genomic and Transcriptomic Hallmarks of Poorly Differentiated and Anaplastic Thyroid Cancers

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J Clin Invest 2016;126(3):1052-1066

Abstract

Background

Poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC) are rare and frequently lethal tumors that so far have not been subjected to comprehensive genetic characterization.

Methods

We performed next-generation sequencing of 341 cancer genes from 117 patient-derived PDTCs and ATCs and analyzed the transcriptome of a representative subset of 37 tumors. Results were analyzed in the context of The Cancer Genome Atlas study (TCGA study) of papillary thyroid cancers (PTC).

Results

Compared to PDTCs, ATCs had a greater mutation burden, including a higher frequency of mutations in *TP53*, *TERT* promoter, PI3K/AKT/mTOR pathway effectors, SWI/SNF subunits, and histone methyltransferases. *BRAF* and *RAS* were the predominant drivers and dictated distinct tropism for nodal versus distant metastases in PDTC. *RAS* and *BRAF* sharply distinguished between PDTCs defined by the Turin (PDTC-Turin) versus MSKCC (PDTC-MSK) criteria, respectively. Mutations of *EIF1AX*, a component of the translational preinitiation complex, were markedly enriched in PDTCs and ATCs and had a striking pattern of co-occurrence with *RAS* mutations. While *TERT* promoter mutations were rare and subclonal in PTCs, they were clonal and highly prevalent in advanced cancers. Application of the TCGA-derived BRAF-RAS score (a measure of MAPK transcriptional output) revealed a preserved relationship with *BRAF*/*RAS* mutation in PDTCs, whereas ATCs were *BRAF*-like irrespective of driver mutation.

Conclusions

These data support a model of tumorigenesis whereby PDTCs and ATCs arise from well-differentiated tumors through the accumulation of key additional genetic abnormalities, many of which have prognostic and possible therapeutic relevance. The widespread genomic disruptions in ATC compared with PDTC underscore their greater virulence and higher mortality.

Introduction

A comprehensive investigation of the genomic landscape of papillary thyroid carcinomas (PTC), the most common thyroid malignancy, was recently reported by The Cancer Genome Atlas Network (TCGA Network) (1). These well-differentiated tumors were found to have a low frequency of somatic alterations (2), with the majority harboring mutually exclusive activating mutations in *BRAF* (60%) and *RAS*-family genes (13%), as well as fusion oncoproteins, primarily involving receptor tyrosine kinases (RTKs) such as *RET*, *NTRK1* or *-3*, and *ALK*. Distinct signaling and transcriptomic consequences were observed between BRAFV600E-like tumors, which showed higher MAPK transcriptional output and lower expression of genes involved in iodine metabolism, and RAS-like tumors, which had lower MAPK signaling and comparatively preserved expression of iodine-related genes.

The TCGA study excluded poorly differentiated thryoid cancers (PDTCs) and anaplastic thyroid cancers (ATCs) from their analysis in order to focus on a homogeneous histological cohort that would provide sufficient power to identify low-frequency genomic events. Although PDTCs and ATCs account for approximately 5%–10% of thyroid cancers, they represent a major clinical challenge. Patients with PDTC and ATC have a mean survival after diagnosis of 3.2 and 0.5 years, respectively, and account for approximately a third of deaths caused by this disease (3). Virtually all cases are refractory to radioiodine therapy, and traditional chemotherapy and radiotherapy are of marginal benefit (4, 5). Molecularly targeted approaches are being tested in preclinical studies and in early human clinical trials (6, 7). These efforts are constrained by the paucity of information on the genomics of these cancers, which have been investigated primarily through Sanger sequencing of a limited set of candidate genes (8–20). The exception to this is a recently reported whole exome sequencing study of ATC (21), which, although informative, may have undercalled significant mutations — particularly those that are subclonal — because of low tumor purity. This is because ATCs, and to a lesser extent PDTCs, pose a particular challenge for genomic studies due to their extensive infiltration by macrophages (22, 23). This cannot be overcome by microdissection of tissue samples because the macrophages form an interconnected network that envelops the individual tumor cells throughout the tumor specimen. To overcome this, we adopted an ultradeep sequencing strategy using the MSK-IMPACT cancer exome panel, a massively parallel exon capture approach that targets all exons and selected introns of 341 genes frequently altered in human cancer (24). We performed this extensive cancer gene exome sequencing as well as expression profiling in the largest series of PDTC and ATC ever investigated, and we identified a wide spectrum of somatic mutations, genetic fusions, and copy number alterations (CNAs) that clearly delineate profound genomic differences between the two advanced forms of the disease. Moreover, when analyzed in the context of the PTC TCGA study (1), this study provides insights into tumor microevolution, suggesting that PDTCs and ATCs evolve from their well-differentiated counterparts.

Results

Samples, clinical data, and overall approach

One-hundred and seventeen advanced thyroid tumors, including 84 PDTCs and 33 ATCs, met the sequencing quality standards and are reported in this study. Clinicopathological features are summarized in Table 1 and Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI85271DS1). Median age was 58 and 66 years for PDTC and ATC, respectively. Female/male ratios were 1.5:1 (PDTC) and 1.2:1 (ATC), which are distinct from PTC (2.7:1) (25). Most samples were primary tumors: 92/117 (64/84 PDTC; 28/33 ATC), and the remainder from nodal (6) or distant (19) metastases.

MSK-IMPACT, a next-generation sequencing (NGS) platform for targeted sequencing of 341 cancer genes (24), was performed in all 117 tumors. A subset of 37 (17 PDTCs and 20 ATCs) was also subjected to an mRNA expression array and a CGH platform to validate IMPACT-derived copy number calls, as detailed later. Median tumor purity was 72% and 42% for PDTCs and ATCs, respectively, consistent with the known heavy macrophage infiltration in ATCs and highlighting the need for deeper sequence coverage in these tumors (Figure 1B). Average depth of coverage was 584× for tumors and 236× for paired normal tissues. Average coverage was 500× and 765×, for FFPE and frozen tumors, respectively. Coverage for ATCs was 739x.

Table 1 | Summary of the clinicopathological features of the 117 advanced thyroid tumors included in the study

		PDTC	ATC
Number of tumors		84	33
Sample type [N (%)]	Primary	64 (76.2)	28 (84.9)
	Metastasis	16 (19.0)	3 (9.0)
	Recurrence in neck	4 (4.8)	2 (6.1)
Age [years]	Median	58	66
	Range	22–87	34–82
Sex [N (%)]	Female	51 (60.7)	18 (54.5)
	Female/Male ratio	1.5:1	1.2:1
Distant metastasis [N (%)]	No	43 (51.2)	0 (0.0)
	Yes	30 (35.7)	15 (45.5)
	lung	16 (19.0)	10 (30.3)
	bone	5 (6.0)	2 (6.1)
	lung + bone	7 (8.3)	2 (6.1)
	other	2 (2.4)	1 (3.0)
	unknown	11 (13.1)	18 (54.5)
Survival [N (%)]	Alive	57 (67.9)	11 (33.3)
Sample preservation [N (%)]	Frozen	17 (20.2)	20 (60.6)
	FFPE	67 (79.8)	13 (39.4)
Normal tissue [N (%)]	Paired normal	78 (92.9)	28 (84.8)
Cytological phenotype [N (%)]	Papillary	32 (38.1)	7 (21.2)
	Follicular	18 (21.4)	0 (0.0)
	Tall cell	19 (22.6)	5 (15.2)
	Hurthle	9 (10.7)	1 (3.0)
	Mixed/other	5 (6.0)	2 (6.1)
	N/A	1 (1.2)	18 (54.5)
PDTC growth pattern [N (%)]	Solid	53 (63.1)	
	Papillary	23 (27.4)	
	Mixed/other	7 (8.3)	
	N/A	1 (1.2)	
PDTC definition [N (%)]	Turin proposal	52 (61.9)	
	MSKCC	31 (36.9)	
	N/A	1 (1.2)	

Somatic mutations

ATCs harbored a higher number of mutations than PDTCs (median ± interquartile range [IQR]: 6 ± 5 and 2 ± 3, respectively, $P < 1 \times 10–4$) (Figure 1A). The mutation burden in PDTCs was increased compared with the PTCs from the TCGA study (considering mutations in the 341 gene set only): 2 ± 3 and 1 ± 1, respectively ($P < 1 \times 10–4$). These differences remained highly significant after removing tumors with defects in mismatch excision repair (MMR) genes, which showed a disproportionately higher number of mutations. Mutation burden in PDTCs (expressed as number of mutations below, equal, or above the median) was greater in older patients (47 vs. 58 vs. 64 years, $P < 1 \times 10–3$) and associated with tumor size (36% vs. 43% vs. 71% > 4 cm, $P = 0.04$),

presence of distant metastasis (8% vs. 29% vs. 57%, P = 2 × 10–3), and overall survival (19% vs. 25% vs. 46%, logrank P = 0.01) (Supplemental Table 2).

Drivers and frequently altered genes. BRAFV600E mutations were present in 33% of PDTCs and 45% of ATCs, whereas mutations in *NRAS*, *HRAS*, or *KRAS* occurred in 28% and 24% of PDTCs and ATCs, respectively, and were mutually exclusive with *BRAF* and gene fusions (Figure 1, C and D, and Supplemental Tables 3 and 4). There was a dichotomy in the distribution of *RAS* and *BRAF* mutations of PDTCs according to their histological features. Ninety- two percent of *RAS* mutations were found in PDTCs fulfilling the Turin definition of the disease (PDTC-Turin; see Methods, ref. 26). By contrast, 81% of *BRAF* mutations were found in PDTCs defined based only on MSKCC criteria (PDTC-MSK): high mitotic rate and necrosis irrespective of growth pattern (ref. 27 and Figure 1, B and C). *BRAF*-mutant PDTCs were smaller and had higher frequency of nodal metastases, whereas their *RAS*-mutant counterparts were larger and had a higher rate of distant metastasis (Supplemental Table 5). *BRAF*-mutant PDTCs were significantly overrepresented among female patients (P = 0.005).

Neurofibromin 1 gene (*NF1)* mutations were identified in 3 *BRAF/RAS* WT ATCs. There were also low-frequency mutations in *TSHR* and *STK11* in both PDTC and ATC (Figure 1C). Mutations in *PIK3CA* and *PTEN*, which encode key effectors of the PI3K/AKT pathway, were particularly prevalent in ATCs (18% and 15%, respectively, Supplemental Table 3) and were overrepresented with respect to PDTCs (P = 4 × 10–2 and 6 × 10–3, respectively). *PIK3CA* and *PTEN* showed distinct patterns of co-occurrence in ATCs. All 3 ATCs harboring *NF1* mutations also had truncating alterations in *PTEN* (P = 2 × 10–3), whereas *PIK3CA* and *BRAF* mutations tended to co-occur. All 5 *PIK3CA* helical domain mutations (E542K or E545K) occurred in ATCs, whereas the single kinase domain mutation (H1047R) was found in a PDTC (Figure 1C).

TP53 and other tumor-suppressor genes. Inactivation of p53 has been considered as a hallmark of advanced thyroid tumors (14, 30). We found that *TP53* mutations, although highly prevalent in ATCs, were relatively rare in PDTCs (73% vs. 8%, P < 1 × 10–4, Figure 1F), which contrasts with previous reports (refs. 11, 31, and Supplemental Table 8) and constitutes a key distinguishing event in the biology of these tumors. *ATM*, a cell-cycle checkpoint and DNA damage response gene, was mutated in 7% of PDTCs and 9% of ATCs. *ATM*-mutated tumors had a higher mutation burden: median was 5 and 2 in *ATM*-mutant versus WT PDTCs (P = 0.04), and 19 and 5.5 in ATC, respectively (P = 7 × 10–3). A higher mutation burden is consistent with the loss of the canonical function of this checkpoint kinase, which is activated in response to DNA double strand breaks and is required for appropriate DNA repair (32). Infrequent truncating mutations were also found in *RB1*, *NF2*, and *MEN1*.

The EIF1AX-RAS association. Mutations in the eukaryotic translation initiation factor *EIF1AX* were initially discovered in uveal melanomas (28) and were also reported in 1% of PTCs (6/402), largely occurring in a mutually exclusive manner with *BRAF* and *RAS* (1). By contrast, 11% of PDTCs and 9% of ATCs harbored *EIF1AX* mutations (Figure 1C and Figure 2A), which were strongly associated with *RAS* (14/15, P < 1 × 10–4, Figure 2B and Table 2). *EIF1AX* mutations clustered in two regions: the N-terminal domain, as also observed in uveal melanomas (28) and other tumors (Figure 2A), or at a unique splice acceptor site between exons 5 and 6 (p.A113splice), which was the most prevalent abnormality and which has not been reported in other tumor types. The C-terminal p.A113 splice mutation predicts for alternative usage of a cryptic splice acceptor within exon 6, resulting in a 12 amino acid in-frame deletion. Our analysis of RNASeq data from two cases with this mutation in the PTC-TCGA confirms this prediction (not shown). EIF1AX mutations were associated with larger tumors (Figure 2C and Supplemental Table 6).

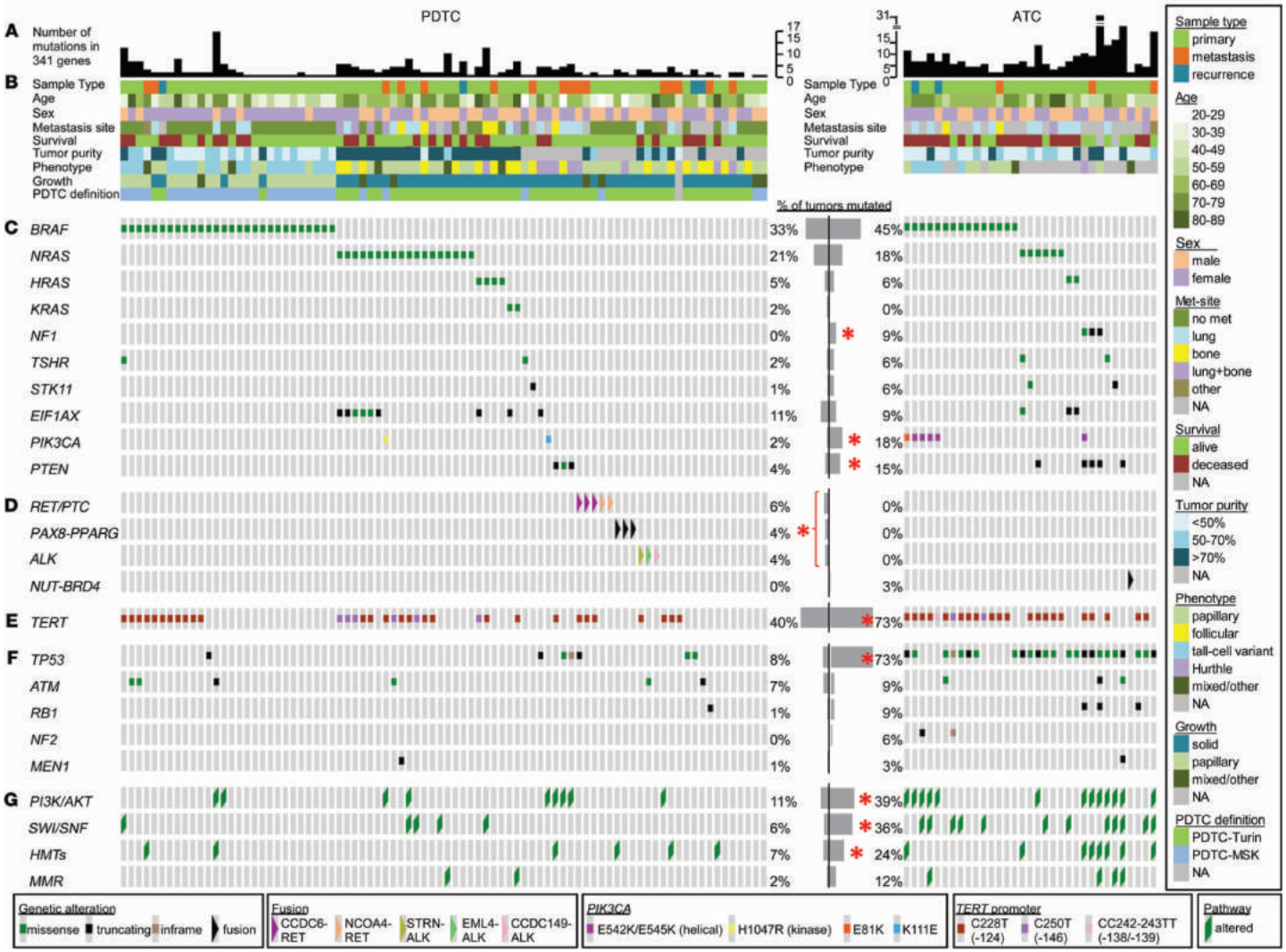
PDTC
ATC
A
Number of mutations in 341 genes
B
Sample Type
Age
Sex
Metastasis site
Survival
Tumor purity
Phenotype
Growth
PDTC definition
% of tumors mutated
C
BRAF 33% 45%
NRAS 21% 18%
HRAS 5% 6%
KRAS 2% 0%
NF1 0% 9%
TSHR 2% 6%
STK11 1% 6%
EIF1AX 11% 9%
PIK3CA 2% 18%
PTEN 4% 15%
D
RET/PTC 6% 0%
PAX8-PPARG 4% 0%
ALK 4% 0%
NUT-BRD4 0% 3%
E
TERT 40% 73%
F
TP53 8% 73%
ATM 7% 9%
RB1 1% 9%
NF2 0% 6%
MEN1 1% 3%
G
PI3K/AKT 11% 39%
SWI/SNF 6% 36%
HMTs 7% 24%
MMR 2% 12%
Sample type
primary
metastasis
recurrence
Age
20-29
30-39
40-49
50-59
60-69
70-79
80-89
Sex
male
female
Met-site
no met
lung
bone
lung+bone
other
NA
Survival
alive
deceased
NA
Tumor purity
<50%
50-70%
>70%
NA
Phenotype
papillary
follicular
tall-cell variant
Hurthle
mixed/other
NA
Growth
solid
papillary
mixed/other
NA
PDTC definition
PDTC-Turin
PDTC-MSK
NA
Genetic alteration
missense
truncating
inframe
fusion
Fusion
CCDC6-RET
NCOA4-RET
STRN-ALK
EML4-ALK
CCDC149-ALK
PIK3CA
E542K/E545K (helical)
H1047R (kinase)
E81K
K111E
TERT promoter
C228T (-124)
C250T (-146)
CC242-243TT (-138/-139)
Pathway
altered

Figure 1 | Cancer genome alterations in 84 PDTCs and 33 ATCs. (**A**) Mutation density across the PDTC and ATC cohorts ($n = 117$), expressed as number of genetic alterations found in 341 genes present in MSK-IMPACT. (**B**) Clinicopathological features, including sample type, patients' age (by decade), sex, metastasis site, survival status, tumor purity, cytological phenotype, growth pattern, and PDTC definition (PDTC-Turin vs. PDTC-MSK). Color keys are shown in the right outermost panel. (**C**–**G**) Oncoprints of PDTCs (left) and ATCs (right). Middle panel shows percentage of tumors altered for each event; *$P < 0.05$ between PDTCs vs. ATCs using Fisher's exact test (see Supplemental Table 3 for extended information). Color key for genetic alterations is shown in the bottom panel. Mutations in drivers and other relevant genes (**C**); fusion events (**D**); *TERT* promoter mutations (**E**); mutations in *TP53* and other tumor suppressor genes (**F**); and alterations in key pathways and functional groups: PI3K/AKT pathway (includes *PIK3CA*, *PTEN*, *PIK3C2G*, *PIK3CG*, *PIK3C3*, *PIK3R1*, *PIK3R2*, *AKT3*, *TSC1*, *TSC2*, and *MTOR*), SWI/SNF chromatin remodeling complex (*ARID1A*, *ARID1B*, *ARID2*, *ARID5B*, *SMARCB1*, *PBRM1*, and *ATRX*), HMTs (*KMT2A*, *KMT2C*, *KMT2D*, and *SETD2*), and MMR (includes *MSH2*, *MSH6*, and *MLH1* genes) (**G**). See Figure 4 for detailed mutational information.

Table 2 | Contingency analysis of *EIF1AX-RAS* mutations in advanced thyroid cancers (PDTCs and ATCs)

	***RAS* WT**	***RAS* mutant**	**Total**
EIF1AX WT	100 (80.6)	24 (19.4)	124 (100.0)
EIF1AX mutant	1 (6.7)	14 (93.3)	15 (100.0)
Total	101	38	139

Number of samples and percentage (in parentheses) of *RAS* mutations in *EIF1AX* WT and mutant samples. The ATC series combines 33 samples from this study and 22 from Kuntsman et al. (21). Statistical analysis show a highly significant co-occurrence of *EIF1AX* and *RAS* mutations (odds ratio = 58.3; 2-tailed Fisher's exact test associated $P < 0.001$).

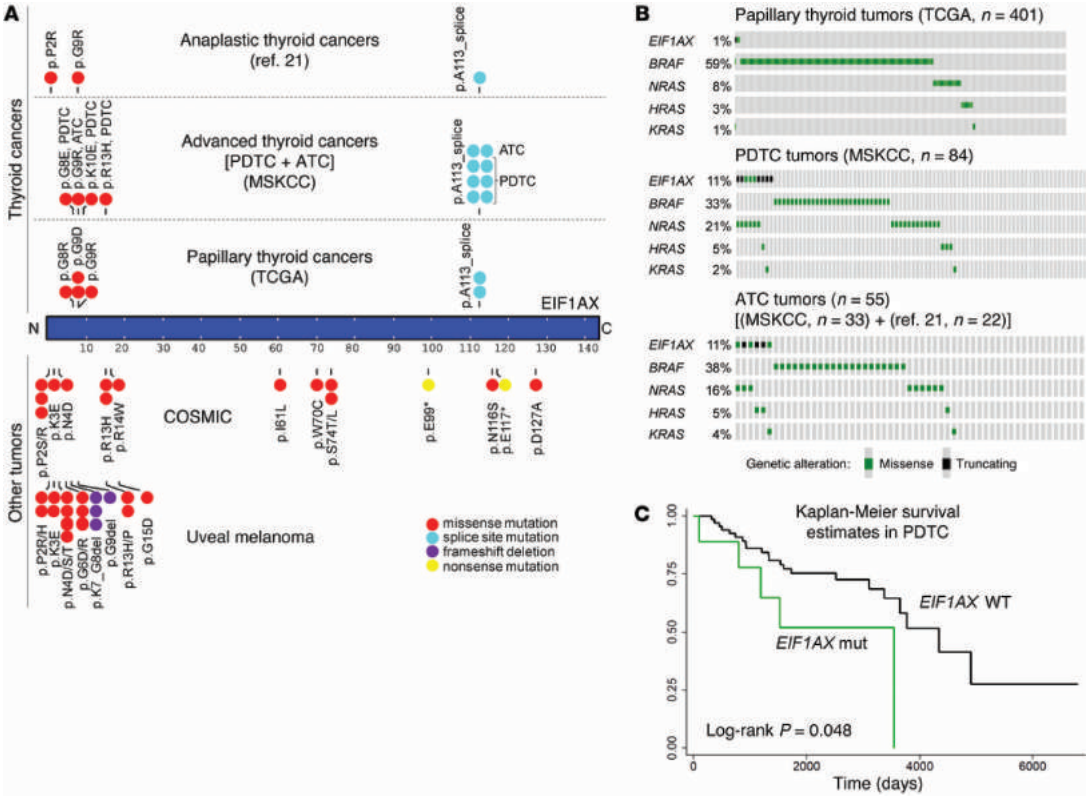

Figure 2 | *EIF1AX* mutations and *EIF1AX-RAS* co-occurrence in thyroid cancers. (**A**) Distribution of *EIF1AX* mutations in thyroid cancers and other tumors (modified from ref. 1). (**B**) Oncoprints showing the co-occurrence of *EIF1AX* with *RAS* mutations in PTC from TCGA (top, $n = 401$), PDTCs from our study (middle, $n = 84$), and ATCs from our series and from (21) (bottom, $n = 55$). (**C**) Kaplan-Meier graph showing significantly shorter survival in *EIF1AX*-mutated PDTCs (log-rank $P = 0.048$). See Supplemental Table 6 for detailed clinical correlations.

TERT in advanced thyroid cancer. There was a high prevalence of telomerase reverse transcriptase (*TERT*) promoter mutations in these advanced thyroid tumors, and their presence was associated with aggressive and metastatic phenotypes (Figure 1E). Together, 40% of PDTCs and 73% of ATCs harbored *TERT* promoter mutations (49/117 C228T [c.-124G>A]; 8/117 C250T [c.-146G>A]) as compared with 9% of PTCs from TCGA (Figure 3, A and B, and Table 3). Whereas *TERT* promoter mutations were subclonal in the small subset of PTC that harbored them, they were clonal in PDTC and ATCs (Figure 3C). *TERT* mutations co-occurred with *BRAF*/*RAS* mutations in PDTCs and ATCs combined (P = 4 × 10–3, Figure 3B and Table 3), consistent with the proposed mechanism whereby the *TERT* mutations generate de novo binding elements for ETS-family transcription factors activated by MAPK signaling, such as GABPA (29). Survival of ATC patients harboring *TERT* promoter mutations was markedly diminished (732 vs. 147 days, P = 0.03, Supplemental Table 7), particularly in cancers with coexisting mutations of *BRAF* or *RAS* (Figure 3D). *TERT*-mutated PDTCs developed more distant metastases (56% vs. 20%, P = 0.01) and had a trend toward greater mortality (Supplemental Table 7). *RAS*-mutant ATCs with *TERT* (5/8) and *EIF1AX* mutations (3/8) did not overlap (odds ratio = 0.01; P = 0.02), consistent with alternate pathways toward progression to ATC.

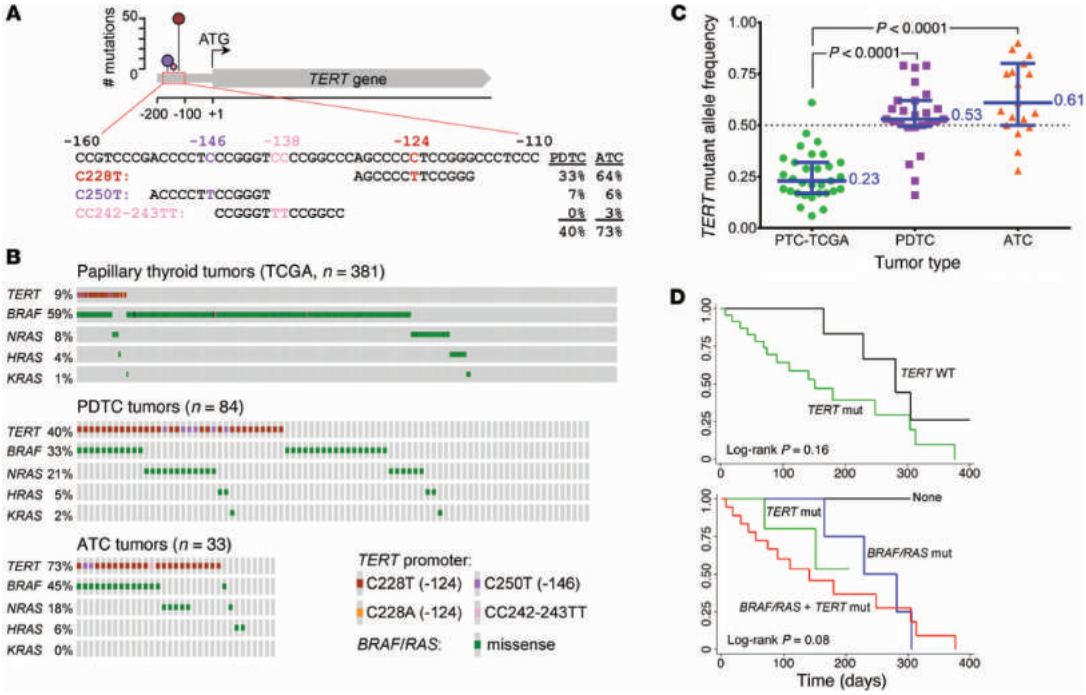

Figure 3 | *TERT* promoter mutations in thyroid cancers. (**A**) Location and overall frequency of *TERT* promoter mutations in PDTCs and ATCs. (**B**) Oncoprints of *TERT* promoter mutations vs. *BRAF* and *RAS* in (top) PTCs from TCGA (*n* = 381); (middle) PDTCs (*n* = 84); and (bottom) ATCs (*n* = 33). (**C**) Allelic frequency of *TERT* promoter mutations in thyroid cancers. Graph shows *TERT* mutant allelic frequency (MAF) corrected for tumor purity, determined based on allelic fraction of driver mutations (*BRAF* or *RAS*) for all three tumor types. (**D**) Kaplan-Meier survival in ATCs with log-rank *P* values. Top: *TERT*-mutant vs WT. Bottom: WT, *TERT*-mutant with or without *BRAF/RAS* mutations. See also Supplemental Table 7.

Table 3 | Contingency analysis of *TERT-BRAF/RAS* mutations in thyroid cancers

	PTC-TCGA[A]			PDTC + ATC[B]		
	***TERT* WT**	***TERT* mutant**	**Total**	***TERT* WT**	***TERT* mutant**	**Total**
BRAF/RAS WT	103	4	107	29	13	42
BRAF/RAS mutant	243	31	2 74	30	45	75
Total	346	35	381	59	58	117

[A]Papillary thyroid cancers from the TCGA study (1), $n = 381$. [B]PDTC and ATC from the current study ($n = 84 + 33$). *TERT* and *BRAF/RAS* mutations significantly co-occur both in PTCs (contingency test: OR = 3.3; $P = 0.03$) and in PDTCs and ATCs combined (contingency test: OR = 3.4; $P = 0.004$). Odds ratios and *P* values are derived from 2-tailed Fisher's exact tests.

Novel genes and pathways altered in advanced thyroid tumors. Mutations of genes encoding members of the PI3K/AKT/mTOR pathway were seen more frequently in ATCs than PDTCs (39% vs. 11%, P = 1 × 10–3). Besides *PIK3CA* and *PTEN*, mutations of *PIK3C2G*, *PIK3CG*, *PIK3C3*, *PIK3R1*, *PIK3R2*, *AKT3*, *TSC1*, *TSC2*, and *MTOR* were also present (Figure 1G, Figure 4, A and E, and Supplemental Figure 1).

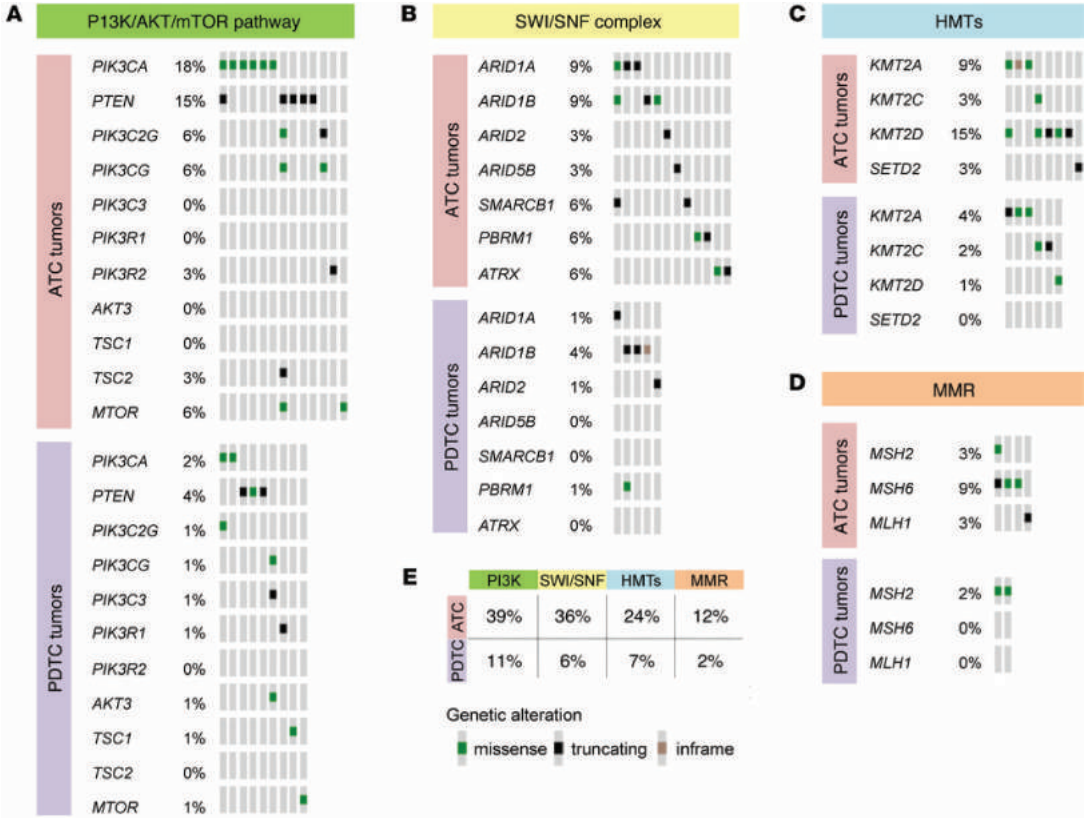

Figure 4 | Pathways and novel functional groups mutated in advanced thyroid tumors. Expanded oncoprints of genes belonging to the indicated functional categories, as defined in Figure 1G. Samples are divided by tumor type (ATC or PDTC) within each panel. Only altered cases, out of 117 tumors, are shown. Missense, truncating, and in-frame mutations are represented as green, black, and brown squares, respectively. (**A**) PI3K/AKT/mTOR pathway (includes *PIK3CA*, *PTEN*, *PIK3C2G*, *PIK3CG*, *PIK3C3*, *PIK3R1*, *PIK3R2*, *AKT3*, *TSC1*, *TSC2*, and *MTOR*); (**B**) SWI/SNF chromatin remodeling complex (*ARID1A*, *ARID1B*, *ARID2*, *ARID5B*, *SMARCB1*, *PBRM1*, and *ATRX*); (**C**) HMTs (*KMT2A*, *KMT2C*, *KMT2D*, and *SETD2*); and (**D**) MMR (*MSH2*, *MSH6*, and *MLH1*). (**E**) Percentage of tumors altered for each functional category and tumor type.

Genes encoding components of the SWI/SNF chromatin remodeling complex were mutated in 36% of ATCs and 6% of PDTCs (P = 1 × 10–4). This is the first report of mutations in *ARID1A*, *ARID1B*, *ARID2*, *ARID5B*, *SMARCB1*, *PBRM1*, and *ATRX* genes in advanced thyroid tumors. Consistent with evidence that disruption of one protein in this complex is typically sufficient to impair function (33), we observed a pattern of mutual exclusivity of mutations in this category of genes (Figure 4B). The single exception was for an ATC with concurrent missense mutations in *ARID1A* (minor allele frequency [MAF] = 5%), *ARID1B* (MAF = 6%), and a frameshift change in *SMARCB1* (MAF = 22%), although their frequencies suggest that they are likely subclonal events within a heterogeneous tumor.

Mutations of the histone methyltransferases (HMTs) *KMT2A*, *KMT2C*, *KMT2D*, and *SETD2* were found in 24% of ATCs and 7% of PDTCs (P = 0.02) (Figure 4C). Additional mutations in chromatin remodeling and epigenetic regulators other than SWI/SNF and HMTs were also seen, including frequent alterations affecting the histone acetyltransferase *CREBBP* and sporadic inactivating mutations in other epigenetic players such as *EP300*, *BCOR*, and *BCL6* (Supplemental Figure 1). Mutations in a few epigenetic regulators (*ARID1B*, *KMT2A*, and *KMT2C*) were also identified in 1%–2% of PTCs from the TCGA, but these events are clearly enriched in advanced thyroid tumors.

Alterations in members of the DNA MMR pathway, including *MSH2*, *MSH6*, and *MLH1*, were found in 12% of ATCs and 2% of PDTCs (Figure 4D). MMR mutant tumors showed a "hypermutator phenotype" (as described in ATCs; ref. 21): median mutation number in MMR-mutant vs. WT ATCs was 16.5 and 5 (P = 1 × 10–3) and, in PDTCs, 7.5 and 2 (P = 9 × 10–3), respectively.

Other genes and functional categories were mutated in a small proportion of PDTCs and ATCs (Supplemental Figure 1 and Supplemental Table 4), i.e., RTKs such as *EPHA3* (3 mutations, exclusively in ATCs), *EGFR*, *FLT1* (*VEGFR1*), *FLT4* (*VEGFR3*), and *KDR* (*VEGFR2*), as well as in all four members of the NOTCH family (NOTCH1–4). Finally, there were infrequent mutations (mutated in at least 2 ATCs or 3 PDTCs) in *DIS3*, *FAT1*, *POLE*, *RBM10*, *RAD54L*, *RECQL4*, and *SF3B1*.

Mutations of genes encoding members of the WNT signaling pathway — i.e., *CTNNB1* (β catenin), *AXIN1*, and *APC* — have previously been reported as genetic hallmarks of ATCs (12, 13), with mutation frequencies >60% for *CTNNB1* (reviewed in Supplemental Table 8). The deep-sequencing results in our cohort do not replicate these findings. We found a single tumor carrying a missense mutation (p.L347P) in *CTNNB1*, which is distinct from the *CTNNB1*–exon 3 gain-of-function hotspot previously reported in this disease. Variants in *AXIN1* were found in 2/117 tumors, both without paired normals available and both of which may have represented low-frequency germline polymorphisms. A single truncating mutation in *APC* (p.Q1529X) was found in an ATC with an unusually high mutation burden. In addition, our results do not support a relevant role for mutations in genes in the apoptosis, Hedgehog, homologous recombination, immune response, insulinlike, JAK-STAT, tricarboxylic acid, nucleotide excision repair, polycomb, ubiquitination, or TGFβ pathways in PDTC and ATC tumorigenesis. With respect to mutations reported in single cases in a recent exome sequencing study of ATCs (21), we replicated these findings in the cyclin-dependent kinase inhibitors *CDKN1B* and *CDKN2C* (and found an additional truncating mutation in CDKN2A), as well as in other genes such as *ERBB2*, *PTCH1*, and *DAXX* (Supplemental Figure 1), but not in *TRAF7* and *NCOR1*.

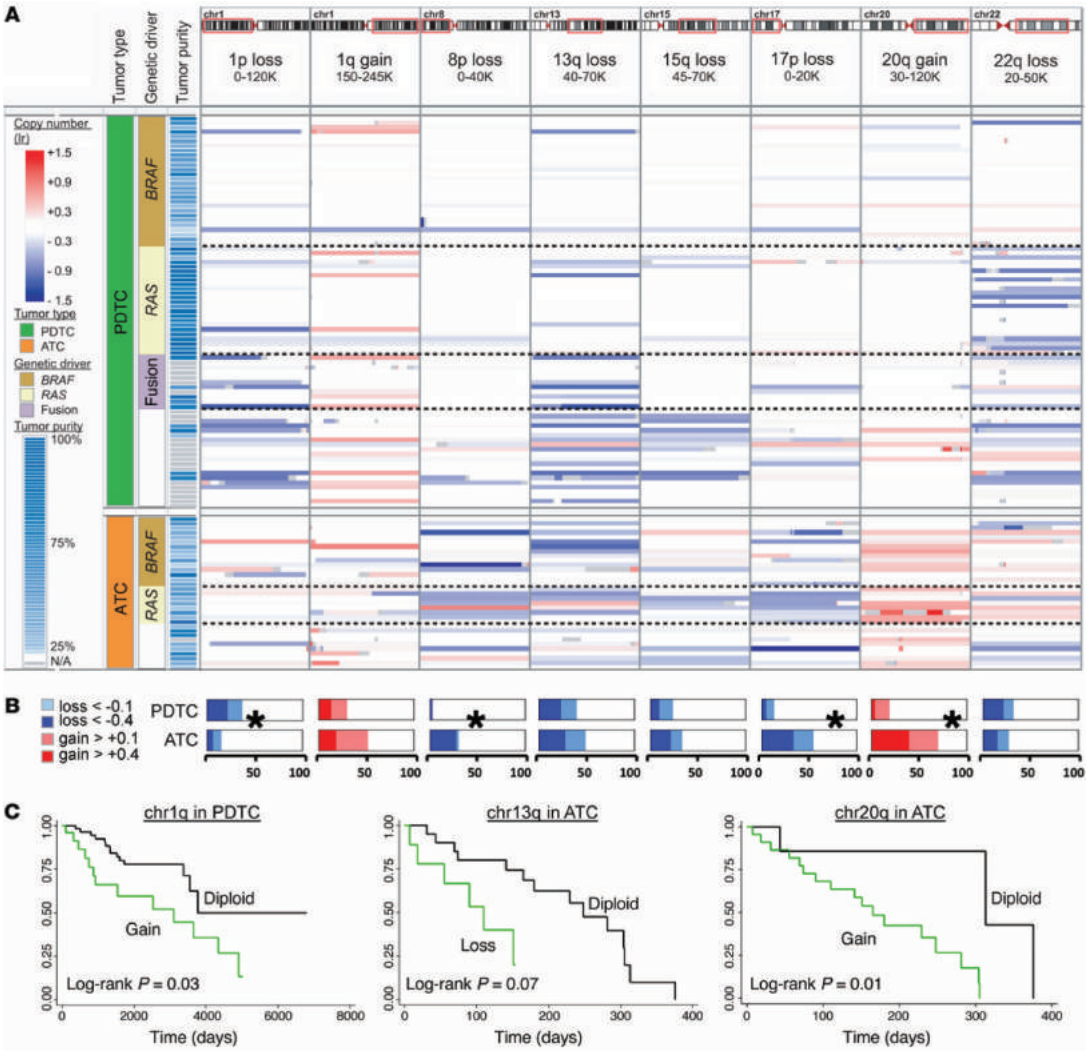

Figure 5 | Recurrent MSK-IMPACT–derived CNAs found in 84 PDTC and 33 ATC. Representation of arm-level regions recurrently gained or lost in PDTCs and/or ATCs. CNAs were corrected for tumor purity in each sample with known driver mutations (see Methods and Supplemental Figure 2). (**A**) IGV representation of the altered chromosomal regions, with approximate locations shown on the top panel (genome build hg19), expressed as red (gain) or blue (loss), with shading intensity proportional to the log-ratio (lr) values. Samples are grouped by tumor type and sorted by genetic driver alteration: *BRAF*, *RAS*, fusions (RET/PTC, PAX8-PPARG, and ALK), or none/unknown. Color key and annotations are shown on the left. (**B**) Frequencies of the indicated CNAs in PDTCs and ATCs. Copy number gains (red) or losses (blue) were defined using two lr thresholds: ±0.1 (lighter shading) and ±0.4 (darker shading). Asterisks denote significant differences expressed as Fisher's exact test *P* values for ±0.4 threshold: PDTC, 0.06 for 1p loss; ATC, $< 2 \times 10^{-4}$ for 8p loss, 17p loss, and 20q gain. (**C**) Kaplan-Meier survival curves for PDTCs harboring chromosome 1q gain (left, log-rank *P* values for ±0.1 and ±0.4 thresholds are 0.03 and 0.06, respectively) and for ATCs with 13q loss (middle, $P = 0.07$ and 0.02) or 20q gain (right, $P = 0.01$ and 0.06).

Gene fusions

Chromosomal rearrangements involving genes known to be translocated in thyroid tumors were frequent events in PDTCs (14%) but were absent in ATCs and did not overlap with *BRAF*, *RAS*, *TSHR*, or *STK11* mutations (Figure 1D). *RET*/*PTC* rearrangements were detected in 5 PDTCs and involved the most common RET partners *CCDC6* and *NCOA4*. Translocations leading to *PAX8*/*PPARG* fusions were observed in 3 PDTCs, whereas

fusions involving *ALK* gene were detected in another 3 tumors. The kinase domain of *ALK* was recombined with three different upstream partners, including the known *STRN* and *EML4*, as well as *CCDC149*, a novel *ALK* fusion partner, which is a coiled-coil family gene located on chromosome 4 (fusion included *CCDC149* exons 1–10 and *ALK* exons 20–29). The 11 PDTCs harboring gene fusions occurred in younger patients (49 vs. 58 years, P = 0.04; Supplemental Table 5). A single ATC without known driver mutations carried a t(15;19)(q13;p13.1) translocation involving the *NUT* gene (*NUTM1*, NUT midline carcinoma, family member 1) and *BRD4* (bromodomain containing 4), resulting in an in-frame *NUT*/*BRD4* fusion (*NUT* exons 1–2 and *BRD4* exons 14–20). It was detected in an ATC with areas of PDTC in a 34-year-old woman who underwent total thyroidectomy and laryngopharyngectomy, plus radiotherapy, and who is alive 10 years after diagnosis. It clearly represents an outlier from the clinical behavior standpoint, which matches with her unique genetic alteration, involved in large-scale chromatin remodeling (34).

Somatic CNAs

Tumor purity of PDTCs was similar to PTCs (median tumor content 74% and 72%, respectively) whereas it was much lower in ATCs (42%) (Supplemental Figure 2). By correcting for tumor purity, we greatly enhanced our sensitivity for detecting CNAs even in most of the heavily infiltrated ATCs, as well as in some PDTCs. The ability of IMPACT to call arm-level CNAs was explored in 37 tumors that were simultaneously profiled by array-CGH. As seen in Supplemental Figure 3, CNA calls were efficiently replicated in both platforms. In addition, IMPACT identified CNAs that were not detected by array-CGH, particularly in heavily infiltrated tumors.

Whereas the genome of PTCs is largely diploid, CNAs in advanced thyroid tumors were common and widespread (Supplemental Figure 4). Interestingly, CNAs were more frequent in the ATCs and PDTCs that lacked driver mutations (Figure 5A). A similar subset of PTCs from the TCGA analysis also possessed a high prevalence of CNAs in the absence of driver mutations (1). Overall, 8 arm-level recurrent CNAs (losses of 1p, 8p, 13q, 15q, 17p, 22q, and gains of 1q and 20q; Figure 5A) were present at higher prevalence, an effect that persisted at a more conservative copy number threshold (see Methods).

Most of the CNAs occurred in a tumor type– and gene context–specific fashion (Figure 5B). Loss of 1p was marginally more frequent in PDTCs (P = 0.06), whereas 8p and 17p losses and 20q gains were far more frequent in ATC genomes ($P < 2 \times 10–4$ for all three). Chromosome 1p, 13q, and 15q losses were enriched in PDTCs without known driver mutations ($\chi 2$ P = 0.03, $2 \times 10–3$, and $3 \times 10–4$, respectively), whereas loss of 22q was strongly associated with *RAS*-mutated PDTCs as compared with *BRAF* tumors ($P = 1 \times 10–3$) (Figure 5A).

Three out of 8 of the recurrent CNAs in the genomes of PDTCs and ATCs were associated with outcome. Patients with PDTCs with gains in chromosome 1q had worse survival (log-rank P = 0.06) (Figure 5C). In ATCs, 13q losses and 20q gains were associated with shorter survival (logrank P = 0.02 and 0.06, respectively).

Gene expression profiling: signaling and differentiation

We used a subset of fresh-frozen specimens of 17 PDTCs and 20 ATCs to derive insights into the gene expression profiles of advanced thyroid cancers. The 37-tumor dataset was representative of the main driver genetic alterations described and included 13 *BRAF*, 12 *RAS*, 5 with alterations in other drivers (*NF1*, *NCOA4*/*RET*, *CCDC149*/*ALK*, *NUT*/*BRD4*, and *STK11*), and 7 without known driver mutations (Supplemental Table 9).

A principal component analysis efficiently separated both entities based on their global gene expression (Figure 6A). Only 2 tumors clustered out of their tumor type group: an ATC from a metastatic specimen (the single one in this subset of 20 ATCs) and a *BRAF*-mutant PDTC that was heavily infiltrated with macrophages (tumor purity = 46%, high M2-macrophage signature score, see also Figure 7A).

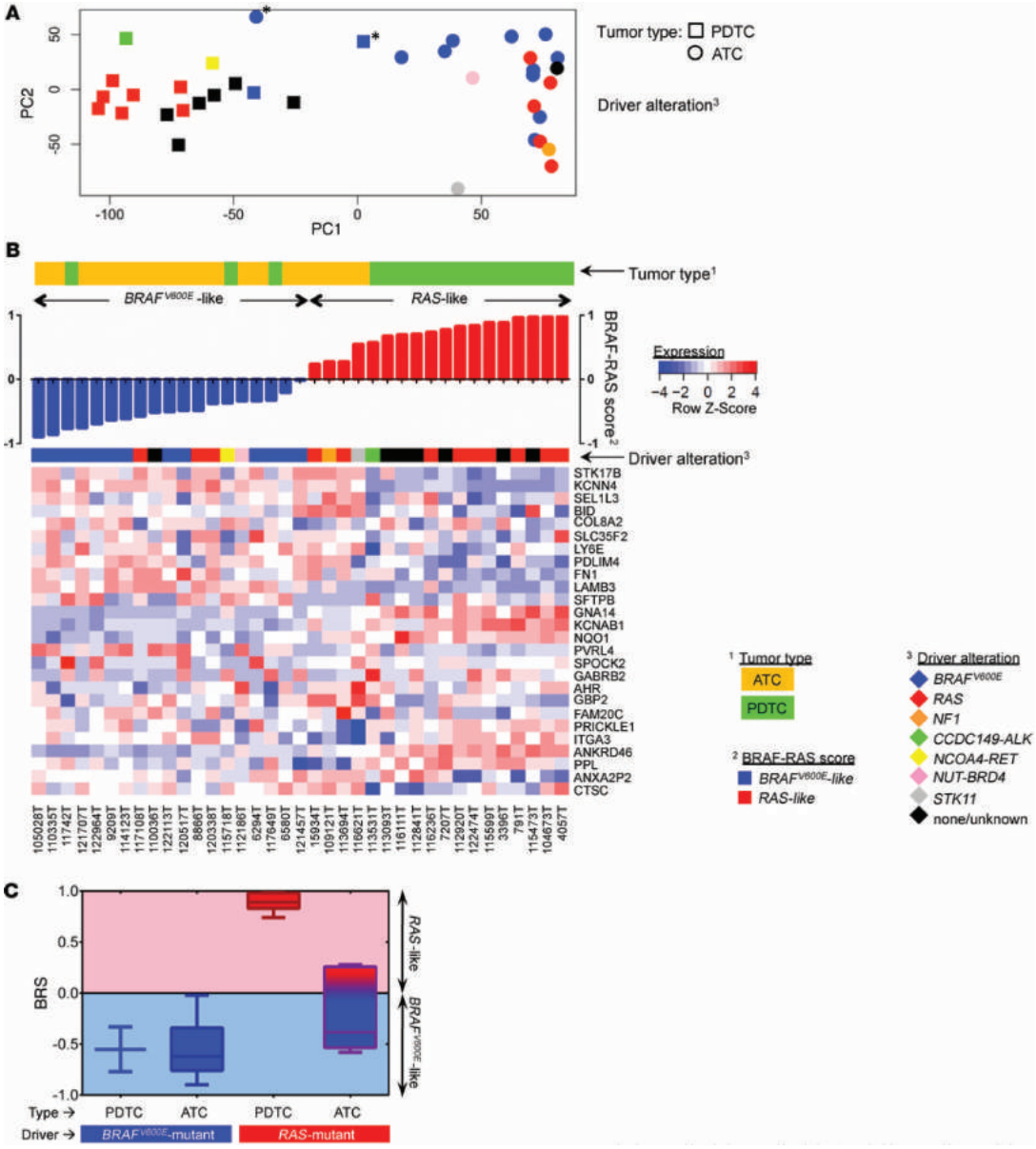

Figure 6 | Principal component analysis (PCA) and BRS of 17 PDTCs and 20 ATCs. (**A**) Two-dimensional PCA discriminates PDTCs (squares) from ATCs (circles). Color-coding for driver alterations is shown in **B**. Asterisks represent ATC and PDTC outliers; see text. (**B**) Heatmap generated by applying the 67-gene BRS signature to advanced thyroid tumors. Expression values are displayed as Z-scores after scaling the values of each gene across the 37 samples. The 26 most informative genes are shown; the complete 67-gene signature is shown in Supplemental Figure 5. Samples are sorted by ascending BRS score: *(BRAF^{V600E}-like* on the left and *RAS*-like on the right) and annotated for tumor type and driver alteration. (**C**) Detailed comparison of driver mutation vs. BRS in *BRAF*- and *RAS-mutant* PDTCs and ATCs. Paradoxically, *RAS-mutant* ATCs are primarily *BRAF^{V600E}-like* (Mann-Whitney *U* test, $P = 0.003$). Box plots were generated using the Tukey method: horizontal lines within each box represent median values, box heights symbolize the IQR (IQR = Q3-Q1); Q3 and Q1 quartiles correspond to the top and bottom boundaries of the box, respectively; whiskers represent values up to 1.5 times IQR greater than Q3 (top: Q3 + 1.5 × IQR) or smaller than Q1 (bottom: Q1 - 1.5 × IQR).

MAPK signaling: the BRAF-RAS score. As a consequence of their genomic simplicity, the key oncogenic drivers of PTCs are associated with distinct biological, signaling, and gene expression properties. Pertaining to this study, the recently published TCGA analysis of 390 PTCs showed clear gene expression differences between *BRAF* and *RAS* tumors, which were used to construct a BRAF-RAS score (BRS) (1). We aimed to evaluate whether these driver-dependent gene expression characteristics persist in advanced cancers, which harbor a more complex cancer genome. Sixty-seven out of the 71 genes in the BRS were present in the mRNA array and were assessed in our PDTCs and ATCs with known driver mutation status. We found a high correlation between BRS values and *BRAF*/*RAS* mutation status (Figure 6, B and C). All 13 BRAFV600E-mutated PDTCs and ATCs were BRAF-like. However, although *RAS*-mutant PDTCs were strongly RAS-like, *RAS*-mutant ATCs were BRAF-like ($P = 3 \times 10–3$), suggesting that a high MAPK transcriptional output is a characteristic property of ATCs, regardless of the driver mutation (Figure 6C and Supplemental Figure 5).

Macrophage infiltration. ATCs are known to be extensively infiltrated with macrophages (22, 23). Although it is assumed that these are M2 macrophages, which promote tumorigenesis, this has not been proven. We applied a previously characterized signature (35) of 78 genes overexpressed in M2 macrophages (68 of which were represented in our array) to the 37 tumors (Figure 7A and Supplemental Figure 6) and found that it was sufficient to discriminate ATCs from the great majority of PDTCs, which are less prone to macrophage infiltration. The only 3 PDTCs that clustered with ATCs had a lower median estimated tumor purity than the rest of the PDTCs, suggestive of significant stromal contamination (46% vs. 84%, respectively; median purity in ATCs was 36%). Consistent with this, the prevalence of likely clonal mutations in ATCs, such as *BRAF* and *TERT*, is higher in this study than that described in prior reports (refs. 10, 17–20, 36–38, and Supplemental Table 8), probably due to IMPACT's deeper coverage.

Thyroid differentiation score. Loss of expression of thyroid differentiation markers is one of the hallmarks of advanced thyroid cancers and has profound consequences for the clinical management of these patients, who are usually refractory to radioiodine therapy due to loss of expression of NIS (sodium iodide symporter, *SLC5A5*) and other genes required for iodine incorporation. The TCGA analysis of PTCs used a thyroid differentiation score (TDS) consisting of 16 genes involved in iodine metabolism and thyroid specification to investigate driver-dependent effects on these parameters. We compared the TDS in PDTCs and ATCs with 8 PTCs profiled with the same platform (39). Overall, PDTCs and PTCs did not differ greatly, whereas ATCs had profoundly suppressed mRNA levels for *TG*, *TSHR*, *TPO*, *PAX8*, *SLC26A4*, *DIO1*, and *DUOX2* genes (Figure 7B and Supplemental Table 9). None of the tumor types expressed *THRB*, *DUOX1*, *SLC5A5*, or *SLC5A8*. Unsupervised clustering based on the TDS discriminated ATCs from PDTCs (Supplemental Figure 7), with the exception of 3 PDTCs. These clustered with ATCs and corresponded to patients who died of the disease, two of whom were the only BRAFV600E-mutated PDTCs in this subset.

Finally, we evaluated the relationship between TDS, BRS, and the driver mutations of these tumors. As shown in Figure 7C, TDS and BRS were correlated in PDTCs, as tumors with low TDS corresponded with BRAF-like specimens, whereas this correlation was completely lost in ATCs. Similarly, *BRAF*-mutated PDTCs showed a marked decrease in TDS when compared with their *RAS*-mutated counterparts, whereas ATCs were homogeneously undifferentiated (Figure 7D).

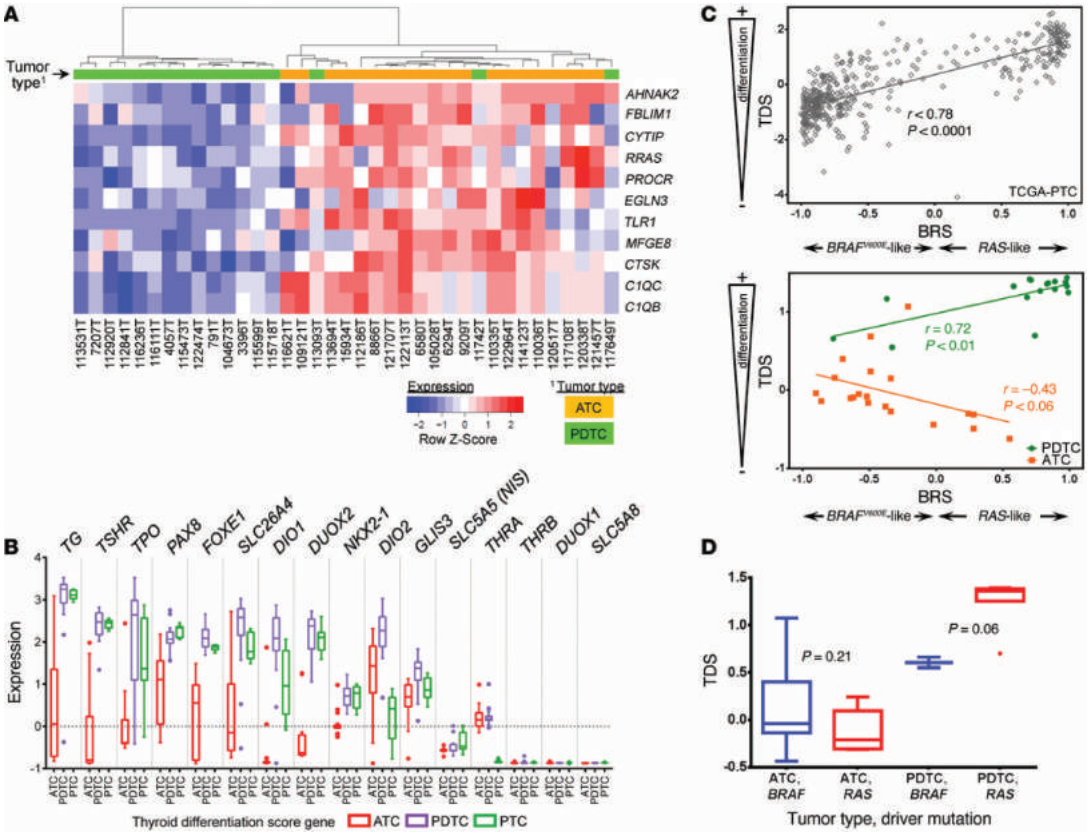

Figure 7 | M2 macrophage signature and TDS of 17 PDTCs and 20 ATCs. (**A**) Unsupervised clustering based on a 68-gene M2-macrophage signature in advanced thyroid tumors (35). Expression values are displayed as Z-scores after scaling the values of each gene across the 37 samples. The 11 most discriminatory genes (variance greater than 2) are shown; the complete 68-gene signature is shown in Supplemental Figure 6. ATCs clearly cluster apart from PDTCs consistent with their extensive macrophage infiltration. (**B**) Relative expression of the 16 genes of the TDS in 20 ATCs and 17 PDTCs, compared with 9 PTCs from He et al. (39) evaluated with the same mRNA array platform. ATCs have low TDS values for virtually all TDS genes, whereas PDTCs are comparable to PTCs. The 16-gene TDS signature discriminates ATCs and PDTCs (see unsupervised clustering in Supplemental Figure 7). (**C**) Correlation plots between TDS and BRS in PTCs from TCGA (top) and PDTCs and ATCs (bottom). Trend lines, Pearson's correlation coefficients (r) and associated P values are shown in the graphs. TDS and BRS are positively correlated in PTCs ($r = 0.74$, $P < 0.0001$) and PDTCs ($r = 0.72$, $P < 0.01$); i.e., RAS-like tumors tend to be more differentiated than BRAF-like cancers. This relationship is lost in ATCs, which are profoundly undifferentiated ($r = -0.43$, $P = 0.06$). (**D**) Comparison of TDS values in *BRAF*- and *RAS*-mutated PDTCs and ATCs. Whereas ATCs are undifferentiated regardless of their driver alteration (Mann-Whitney U test, $P = 0.21$), *BRAF*-mutated PDTCs show a decrease in TDS compared with their *RAS*-mutant counterparts ($P = 0.06$). Box plots from **B** and **D** were generated using the Tukey method: horizontal lines within each box represent median values; box heights symbolize the IQR (IQR = Q3–Q1); Q3 and Q1 quartiles correspond to the top and bottom boundaries of the box, respectively; and whiskers represent values up to 1.5 times IQR greater than Q3 (top: Q3 + 1.5 × IQR) or smaller than Q1 (bottom: Q1 − 1.5 × IQR). Values outside these limits are considered outliers and are represented by dots.

Discussion

Most PDTCs and ATCs are thought to arise from preexisting PTCs based on their frequent co-occurrence in the same tumor specimen, where they consistently share a driver mutation (36, 40). Our results, analyzed in the context of the PTC TCGA study, provide insights into tumor microevolution and lend support to this model of tumorigenesis. Particularly compelling in this regard is the fact that mutations in the *TERT* promoter, which are known to activate its transcription, display a stepwise increase in frequency along the spectrum of disease progression (9% in PTCs, 40% in PDTCs, and 73% in ATCs) (1, 17–20). Interestingly, *TERT* mutations are

subclonal in the few PTCs that harbor them, whereas they are clonal in PDTCs and ATCs, pointing to selection during tumor evolution, possibly by inducing cell immortalization. *TERT* promoter mutations also track with virulence of advanced disease. They are significantly associated with *BRAF* or *RAS* mutations, consistent with the proposed mechanism by which mutations in the promoter, by generating novel consensus motifs for the ETS family of transcription factors, promote *TERT* overexpression in cells with constitutive activation of MAPK signaling.

By using a platform with a depth of sequencing optimized to identify mutations in tumors known to be associated with abundant stromal contamination, primarily by tumor associated macrophages (TAMs) (22, 23), we identified key genetic lesions that distinguish PDTCs from ATCs (i.e., *TP53*, *TERT*, and genes encoding effectors in the PI3K pathway). This includes genetic defects that implicate functional programs not previously associated with thyroid cancer, such as the SWI/SNF complex, HMTs, and others.

With respect to the main driver alterations, *BRAF* mutations were less prevalent in advanced tumors compared with PTCs, whereas *RAS* mutations were more frequent. Rearrangements commonly seen in radiation-induced and, to a lesser extent, in sporadic PTCs (*RET*/*PTC*, *PAX8*/*PPARG* and *ALK* fusions) were present in a subset of PDTCs but absent in the ATCs we sampled (9, 41, 42). *NF1* mutations were only found in ATCs in our series. The TCGA study of PTCs showed that *BRAF*- and *RAS*-mutant tumors exhibited profound differences in their clinical and histological characteristics and in their gene expression profile. The BRS is a 71-gene panel that distinguishes BRAFV600E from *RAS*-mutant PTCs. It was highly correlated to the transcriptional output of the MAPK pathway, which was highest in *BRAF*-mutant cancers. This is explainable because ERK activation in *RAS*-mutant cells induces a negative feedback that disrupts RAF dimerization, thus attenuating pathway output. By contrast, BRAFV600E signals as monomer and is unresponsive to this constraint, resulting in a greater flux through the pathway (1, 43). We found that these sharp demarcations between *BRAF*- and *RAS*-mutant disease persisted in PDTC but were largely lost in ATC. PDTCs that met the standard histological definition of that entity (Turin proposal, ref. 26) were strongly associated with *RAS* mutations. By contrast, those defined based on the presence of high mitotic rate and necrosis irrespective of other characteristics (27) were markedly enriched for *BRAF*. They also had distinct clinical behaviors: *BRAF*-mutant PDTCs primarily developed locoregional nodal metastases, whereas *RAS*-mutant PDTCs presented with distant metastases. The BRS tracked with the underlying driver mutation in PDTCs but not in ATCs. This was also true for a score derived from a gene set consisting of mRNAs encoding proteins required for the differentiated function of thyrocytes (the TDS). The greater genomic complexity of ATCs may account for blurring the association of gene expression with the nature of the underlying driver mutation, in particular because of the higher frequency of mutations of genes encoding chromatin modifiers or genes that activate parallel pathways. Interestingly, even ATCs with *RAS* or other mutations tend to be BRAF-like, as defined by the BRS. In addition, ATCs are extensively infiltrated by TAMs. Accordingly, nonhierarchical analysis of a gene set that defines M2 macrophages clearly separated ATCs from PDTCs. It may be that the greater cellular heterogeneity of ATCs may account in part for the attenuation of the oncogenic driver effects on gene expression.

EIF1AX, which encodes for a key component of the translation preinitiation complex (PIC), is mutated in only 1% of PTCs but in approximately 10% of PDTCs and ATCs. The concordance of *EIF1AX* with *RAS* mutations is extremely strong, which is distinct from PTC, where these are largely mutually exclusive. The biological consequences of this association are currently unknown. The mutations of *EIF1AX* cluster in specific N- and C-terminal residues. The C-terminal p.A113 splice mutation is specific to thyroid cancer and predicts for alternative usage of a cryptic splice acceptor within exon 6, resulting in a 12–amino acid in-frame deletion. Our analysis of RNASeq data from two cases with this mutation in the PTC TCGA confirms this prediction (not shown). EIF1A plays a key role in regulating the conformation of the PIC and in scanning for the AUG initiation

codon, which is disrupted in distinct ways by N-terminal and C-terminal mutations in yeast (44). Interestingly, *EIF1AX* mutations are mutually exclusive with alterations in any of the PI3K/AKT/mTOR pathway members, suggesting that they may confer overlapping functional gains. In addition, *EIF1AX* mutations are predictive of worse survival in PDTCs, providing a potentially useful marker for risk stratification in a heterogeneous disease in need of better prognostic indicators (45, 46).

Both entities differed in overall mutation burden, which was significantly higher in ATCs. Within PDTCs, a higher number of mutations was associated with larger tumors, presence of metastasis, and shorter survival. *TP53* mutations, in particular, distinguished ATCs from PDTCs (73% vs. 8%, respectively). CNAs, some of which had been previously reported at very low frequencies (47–51), proved to be distinctive of each tumor type. PDTCs have a greater frequency of 1p losses, whereas 8p and 17p losses, as well as 20q gains, were more common in ATCs. Interestingly, 22q losses were strongly asso- ciated with *RAS*-mutant PDTCs. Loss of the 22q tumor suppressor gene *NF2*, which encodes for merlin, has been recently implicated in this association. Consistent with this, the combined activation of oncogenic Ras with Nf2 loss leads to development of PDTC in mice. This is because inactivation of the Hippo pathway through merlin loss leads to a YAP-TEAD– dependent transcriptional activation of oncogenic and WT *RAS*, thus enhancing MAPK transcriptional out- put and promoting transformation (1, 52).

We compared the results of our targeted cancer gene NGS (IMPACT) approach with a recently reported whole exome sequencing (WES) study of 22 ATCs (21). The greater depth of sequencing achieved by IMPACT (739× vs. 264× in the WES study) may explain the differences observed between both platforms in the frequency of *TP53* (73% by IMPACT vs. 27% by WES), *BRAF* (45% vs. 27%), *PIK3CA* (18% vs. 9%), and *PTEN* (15% vs. 0%) and may improve detection of subclonal events. This is particularly relevant in ATCs because of their low tumor purity, which calls into question the suitability of WES as the platform of choice. Moreover, the WES approach failed to detect mutations in members of the SWI/SNF and HMT functional groups that we report here and that likely play a fundamental role in the biology of these tumors. We acknowledge, however, the drawbacks of sequencing a limited set of cancer genes. For instance, *RASAL1*, *USH2A*, *HECTD1*, *MLH3*, and *MSH5*, which were rarely mutated in ATCs by WES, were not included in IMPACT. Two of these, *MLH3* and *MSH5*, are MMR genes, a functional group that we find to be disrupted in at least 12% of ATCs.

The biological consequences of the novel drivers (e.g., *EIF1AX* and *RAS*) and functional groups (SWI/SNF) in the context of thyroid tumorigenesis remain to be explored. Others recapitulate phenotypes observed in genetically engineered mouse models of advanced thyroid cancers, such as *PTEN* and *TP53* (53), *BRAF* and *TP53* (54), *RAS* and *TP53* (55), *RAS* and *NF2* (52), and *BRAF* and *PIK3CA* (56).

The findings reported here provide tools that can be leveraged to improve the molecular diagnosis of these clinical entities, many of which likely have prognostic implications. Particularly relevant is the strong association of PDTC-Turin tumors with *RAS* mutations, whereas PDTC-MSK tumors were strongly associated with *BRAF*. In addition, *RAS*- and *BRAF*-mutant PDTCs have distinct tropism for metastases, with the former tending to home at a distance, whereas the latter metastasize to locoregional lymph nodes. Moreover, the discovery in well-differentiated tumors of subclonal mutations of genes that we show to be enriched in advanced disease should raise particular concerns. This also opens a path to explore the biology of novel genetic associations that may point to tumor dependencies that can be exploited therapeutically.

Supplementary data for this study are available at The Journal of Clinical Investigation Online (https://www.jci.org).

Methods

Patient tissue samples. PDTC and ATC samples were randomly selected from the pathology department files of the institution from 1986–2015. ATCs were classified according to the last WHO classification of endocrine tumors, whereas PDTCs were defined as follows: (i) according to the Turin proposal, by architectural and high-grade features (mitosis and necrosis), the presence of a solid/ nested/insular growth, the absence of nuclear features of PTC, and either convoluted nuclei, mitotic activity ≥ 3 × 10 high power fields (HPF), or tumor necrosis (26); and (ii) as a carcinoma displaying high mitotic activity (≥ 5 mitosis/10 HPF, ×400) and/or tumor necrosis, and showing follicular cell differentiation at the morphological or immunohistochemical level (27). MSK-IMPACT targeted sequencing was performed in all 117 tumors (84 PDTCs and 33 ATCs): 80 from formalin-fixed paraffin-embedded tissues (FFPE) and 37 from fresh-frozen material. The 37 frozen tumors, 17 PDTCs and 20 ATCs, were also expression profiled with Affymetrix U133 plus 2.0 array and with the Agilent SurePrint G3 CGH 1x1M array- CGH platform to validate copy number calls.

Single nucleotide variant and indel calling and filtering. Single nucleotide variants (SNVs) and short indels (<30 bp in length) were automatically annotated by the MSK-IMPACT pipeline, as previously described (24). Tumor samples without paired normals (11/117; 5 ATCs and 6 PDTCs) were compared against pooled normals. All variants were annotated based on the information available in catalog of somatic mutations in cancer (COSMIC; http:// cancer.sanger.ac.uk/ cosmic), NCBI-dbSNP (http://www.ncbi.nlm.nih.gov/snp), and the 1,000 Genomes Project (http://www.1000genomes.org/). Variants highlighted in this study were subsequently manually reviewed. For the 106 tumors with paired normals, all variants confirmed as somatic were reported, regardless of location and clonality. For tumors compared against pooled normals, MSK-IMPACT automatically called SNVs with reported frequencies <1%.

We manually reviewed the 11 tumors fulfilling these criteria as follows: (i) keeping variants reported in COSMIC; (ii) removing variants reported as polymorphic (with an reference sequence code in dbSNP); and (iii) removing variants with allele frequencies that were >10% of the allelic fraction of the driver mutation in the same tumor.

MSK-IMPACT sequencing data is publicly available at the cBio- Portal for Cancer Genomics (http://www.cbioportal.org/). Mutation plots were generated using the OncoPrinter (v1.0.1) and Mutation- Mapper (v1.0.1) tools, which are available at the cBioPortal (57, 58).

Chromosomal rearrangements were called for genes whose introns were covered by MSK-IMPACT, which included most of the previously reported fusions in thyroid tumors (with the notable exceptions of *NTRK1* and *NTRK3*).

CNAs and estimation of tumor purity. DNA CNAs were primarily called from IMPACT by comparing sequence reads of targeted regions in tumors relative to a standard diploid normal sample, as described (24). Although IMPACT targets a discrete number of exons in each chromosome arm, it efficiently identified arm-level chromosomal genetic gains and losses, as confirmed by a genome- wide Agilent SurePrint G3 CGH 1x1M array-CGH platform in a subset of 37 advanced thyroid tumors (Supplemental Figure 3), which showed excellent agreement in the copy number calls between the two methodologies.

As the macrophage infiltration of advanced thyroid tumors (particularly ATCs) can impact the sensitivity of CNA detection, we corrected CNA values for each tumor based on tumor purity. Tumor purity was calculated based on the mutant allele frequencies of clonal heterozygous somatic mutations in regions lacking overt CNAs (see also Supplemental Figure 2). For tumors with a *BRAF* or *RAS* mutation (heterozygous mutations considered clonal events) (59), purity was calculated by doubling the frequency of the alternate allele (e.g., in a tumor with

a BRAFV600E mutation frequency of 0.45, tumor purity was 90%). For tumors without *BRAF* or *RAS* mutations, we used other gene mutation frequencies as purity estimators In tumors lacking canonical drivers, we used an average of all likely clonal events. We discarded likely subclonal events (e.g., mutations with disproportionately low allelic frequencies compared with other somatic variants). We discarded tumor suppressors (i.e., *TP53*, *NF1*) as purity estimators due to possible coexisting loss-of-heterozygosity events. Chromosomal regions harboring the drivers (typically *BRAF* or *RAS*) were confirmed to be diploid before using those genes as purity estimators. Altogether, we derived tumor purity data from 75 *BRAF*/*RAS*-mutated tumors and used alternative genes for 19 others. Purity could not be estimated with confidence for 23 tumors (22 PDTCs and 1 ATC).

Segmented genome-wide copy number was corrected for tumor purity in the following manner. For all segments where r is the $\log_2$ ratio of segmented copy number, then R = 2r and α is the estimated tumor purity, the copy ratio in cancer cells of a cellularly heterogeneous sample is therefore: R′ = (R/a) – ([1 – a]/a) for all r > $\log_2$(1 – a), otherwise R′ = (r/min[r]) min (R′).

The final purity-corrected copy number is $\log_2$(R′) for all segments in the specimen. CNAs were determined using the purity-corrected segmented data utilizing one of two thresholds (±0.1 and the more conservative ±0.4 threshold). All analyses were expressed at ±0.4, with the exception of survival analyses. As focal events were few, the analyses described here focused on larger and overt arm-level CNAs. Given the challenge of definitively establishing thresholds of heterozygous loss and homozygous deletions in impure tumors (signal compression), our analysis excluded homozygous CNAs. CNAs were visualized in the Integrative Genomics Viewer (IGV), version 2.3.57 (https://www.broadinstitute.org/igv/) (60).

Gene expression. We performed mRNA expression on 37 tumors using the Affymetrix U133 plus 2.0 array. Expression normalization was performed using the gcRMA method (61).

Statistical analyses were performed using the R statistical programming language (62). The lists of genes for the BRS and TDS scores were obtained from the TCGA study on PTC (1). Of the 71 BRS genes, 67 were present on our array and were used to compute the BRS score as described (1). We performed sample-wise scaling on the gene-expression profiles of the 9 PTC tumors, which were derived from a published study (39). The lists of differentially expressed genes between M1 and M2 macrophages (35) were from the MSigDB (63). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (64) and are accessible through GEO Series accession number GSE76039.

Clinical associations. Statistical analyses were performed using STATA/IC (version 12; StataCorp LP). Pathological characteristics were only available for resected ATCs. PDTCs had complete clinical and pathological data. Survival was recorded as of April 2015. All associations reported in PDTCs remained significant when only considering PDTCs based on the Turin criteria definition.

Statistics. Distribution of mutation frequencies in PDTCs versus ATCs was assessed by Fisher's exact tests. Copy number distribution between multiple groups was evaluated with $\chi 2$ tests. Statistical analyses and graphic representations of mutation and CNA distribution were performed on GraphPad Prism 6.02 (GraphPad Software). Demographic and clinicopathologic characteristics were compared using Pearson $\chi 2$ test for categorical variables and 2-tailed Student's t test or Mann-Whitney U test for continuous variables. Survival analyses were conducted with Cox proportional hazards models. Proportionality assumptions were tested with Schoenfeld residuals and log-log plots. Kaplan-Meier survival curves were built, and the logrank test was used to assess for significance of the surviving function. A *P* value less than 0.05 was considered significant.

Study approval. The study was approved by the Institutional Review Board of MSKCC.

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Chapter 9

Genomic Alterations in Fatal Forms of Non-anaplastic Thyroid Cancer: Identification of *MED12* and *RBM10* as Novel Thyroid Cancer Genes Associated with Tumor Virulence

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Clin Cancer Res 2017; 23(19): 5970-5980

Abstract

Purpose

Patients with anaplastic thyroid cancer (ATC) have a very high death rate. In contrast, deaths from non-anaplastic thyroid (NAT) cancer are much less common. The genetic alterations in fatal NAT cancers have not been reported.

Experimental design

We performed next-generation sequencing of 410 cancer genes from 57 fatal NAT primary cancers. Results were compared with The Cancer Genome Atlas study (TCGA study) of papillary thyroid cancers (PTCs) and to the genomic changes reported in ATC.

Results

There was a very high prevalence of *TERT* promoter mutations, comparable with that of ATC, and these co-occurred with *BRAF* and *RAS* mutations. A high incidence of chromosome 1q gain was seen highlighting its importance in tumor aggressiveness. Two novel fusion genes *DLG5-RET* and *OSBPL1A-BRAF* were identified. There was a high frequency of mutations in *MED12* and these were mutually exclusive to *TERT* promoter mutations and also to *BRAF* and *RAS* mutations. In addition, a high frequency of mutations in *RBM10* was identified and these co-occurred with *RAS* mutations and *PIK3CA* mutations. Compared with the PTCs in TCGA, there were higher frequencies of mutations in *TP53*, *POLE*, PI3K/AKT/mTOR pathway effectors, SWI/SNF subunits, and histone methyltransferases.

Conclusions

These data support a model, whereby fatal NAT cancers arise from well-differentiated tumors through the accumulation of key additional genetic abnormalities. The high rate of *TERT* promoter mutations, *MED12* mutations, *RBM10* mutations, and chromosome 1q gain highlight their likely association with tumor virulence.

Introduction

Patients diagnosed with anaplastic thyroid cancer (ATC) have a very high death rate. Such patients have a mean survival after diagnosis of only 6 months. We have recently reported the genomic hallmarks of ATC showing a very high incidence of *TERT* promoter mutations in 73% of cases with a co-occurrence with either *BRAF* or *RAS* mutations (1). We also identified a high rate of mutations in *TP53* (73%) as well as a higher frequency of alterations in genes such as *EIF1AX* (9%), *PIK3CA* (18%), and *ATM* (9%) as compared to those reported by The Cancer Genome Atlas Network (TCGA Network) study of well-differentiated papillary thyroid cancer (PTC), which showed a low frequency of somatic alterations (2).

Non-anaplastic thyroid cancers (NATs) derived from thyroid follicular cells comprise well-differentiated (WDTC) and poorly differentiated thyroid cancer (PDTC). Deaths from WDTC are extremely rare occurring in 1% to 2%, however, because these tumors are the most common form of the disease, this small percentage represents a significant fraction of patient dying from thyroid cancer. Deaths from PDTC are more common, occurring in 30% of patients (3). The genomic characteristics of fatal cases of NAT cancers (FNAT) has not been reported before. We hypothesized that such cancers may harbor genetic similarities to ATC. The objective of our study was to report the genetic alterations in fatal cases of NAT cancer and compare their molecular profile with that of ATC and to the TCGA landscape of PTCs. Fatal cases of NAT cancer are invariably refractory to radioiodine therapy, and traditional chemotherapy and radio-therapy are of marginal benefit (4). Two multikinase inhibitors, sorafenib and lenvatinib, have been approved for treatment of radioiodine refractory NAT cancer. Other drugs are currently being tested in early human clinical trials (5), but these efforts are hindered by the lack of knowledge on the genomics of fatal cases of NAT cancer. The identification of the genetic alterations in these cancers will, therefore, have major implications both in our ability to identify those rare patients at high risk of death and also to develop novel drugs, which target the pathways responsible for their poor prognosis.

Materials and Methods

Patients and tumor samples

After IRB approval, patients with fatal NAT cancer (FNAT) were identified from a database of 3,774 patients who had primary surgery treatment at Memorial Sloan Kettering Cancer Center from 1985 to 2010. Eighty-six (2.3%) patients were identified who had either died from disease or died with disease. Of these, paraffin-embedded tissue blocks from primary tumors were available on 57 (66%) patients. Following Institutional Review Board (IRB) approval, tumor and matched normal (non-neoplastic normal tissue) specimens were obtained, and then hematoxylin and eosin–stained tumor sections were independently re-evaluated by head and neck pathologists (R. Ghossein; D.L. Carlson; B. Xu). Tumors were then classified into PDTC, as defined by histological and/or immunohistochemical evidence of follicular cell differentiation and presence of tumor necrosis and/or 5 mitoses per 10 high-power fields (400; ref. 6) and into well-differentiated thyroid cancer (WDTC). Patients with WDTC were further classified into follicular carcinoma, Hurthle cell carcinoma and the different histological subtypes of papillary thyroid carcinoma such as classical, follicular variant and tall cell variant. Patient demographics, tumor histology, treatment, and outcomes were determined by retrospective review of patient charts. Tumors were staged according to the 7th edition of the AJCC staging manual.

Sequencing platform and variant calling

The dataset comprised 35 PDTC and 22 WDTC tumor samples. All 57 tumors were sequenced using the MSK-IMPACT (Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets) platform, a deep-coverage, targeted next-generation sequencing (NGS) assay encompassing 410 cancer-related genes

and approved for clinical use by the NY State Department of Health (7). Of the 35 PDTC, 20 had previously been sequenced using an earlier iteration of MSK-IMPACT comprising 341 genes and reported as part of a cohort of 84 PDTC (1). The MSK-IMPACT assay is an NGS assay approved for clinical use through CLIA (Clinical Laboratory Improvement Amendments) by the Centers for Medicare and Medicaid Services (8). MSK-IMPACT is optimized for DNA extracted from low-input formalin-fixed, paraffin embedded (FFPE) samples. The assay is designed to detect single-nucleotide variants (SNV), indels, copy-number variants (CNVs), and structural variants in genes that are functionally relevant to cancer and/or clinically actionable targets. The current assay uses hybrid capture technology (NimbleGen SeqCap EZ library custom oligo) to perform deep (>200x) sequencing (Illumina HiSeq 2500) of all 5781 exons and selected introns of 410 cancer genes, including canonical and selected non-canonical transcripts, the *TERT* promoter region, and 33 introns of 14 rearranged genes (Supplementary Table S1). The panel includes 1,042 tiling probes covering single-nucleotide polymorphisms (SNP), allowing genotyping to ensure tumor-normal matching, identify contaminating DNA, and serve as a low-density SNP array for CNV analysis. MSK-IMPACT has been extensively validated.

Copy-number aberrations were identified by comparing sequence coverage of targeted regions in a tumor sample relative to a standard diploid normal sample (7). To call allele-specific somatic DNA copy number, we also applied an integrated pipeline called FACETS (9) to Tumor/Normal pairs of bam files according to authors' recommendations. The bam files were processed to generate a read count matrix for all the potentially polymorphic sites from dbSNP/1000-genome database as well as pseudo SNPs to account for regions that have large gaps between consecutive SNPs. The read counts are then used to compute the GC-corrected normalized log-ratio of tumor to normal read depths for total copy number and log odds ratio from cross-tabulating the tumor and normal reads into ref and alt alleles for loci that are heterozygous in the germline. These are then segmented jointly to obtain the regions of constant allele-specific copy numbers and the segmented data used for allele-specific integer copy-number calls as well as cellular fractions.

Results

Patient, tumor, treatment, and outcome characteristics

Of 57 patients, 52 (91%) patients were over 45 years of age, and 32 (56%) were female. The majority of patients had advanced stage disease; 53 (92%) had pT3 or T4 tumors, 34 (60%) gross extra thyroidal extension, and 31 (54%) had central or lateral neck metastases. Thirty (53%) patients presented with distant metastatic disease. Thirty-five patients had PDTC, and 22 had WDTC of whom two were classical PTC, two follicular variant of PTC, one micro PTC, one PTC with tall cell features, 12 tall cell variant of PTC, and four Hurthle cell carcinoma. Fifty-two patients were treated by total thyroidectomy, and five had less than total thyroidectomy (four lobectomy and one subtotal thyroidectomy). The cause of death was distant metastatic disease in 51 patients, locoregional and distant disease in three patients, and locoregional disease in three patients. The median time to death was 52 months (Table 1).

Somatic mutations

Frequently altered genes. There was a high prevalence of telomerase reverse transcriptase (*TERT*) promoter mutations occurring in 60% of patients (PDTC 60%, WDTC 60%). This high prevalence is comparable to 73% of ATC (1) and far higher than the 9% of PTCs from TCGA (2). *TERT* mutations co-occurred with *BRAF* mutations (18/26 $P<0.001$). They also showed a trend to co-occurrence with *RAS* mutations (10/16) and *EIF1AX* mutations (5/7). *TERT* mutations were mutually exclusive with *TP53* mutations ($P=0.08$) and with *MED12* mutations ($P=0.04$), consistent with alternate pathways toward progression to FNAT (Fig. 1).

Table 1 | Patient, tumor, treatment, and outcome characteristics of 57 patients with fatal NAT cancer

	N **(%)**
Age	
<45 years	5 (9%)
≥45 years	52 (91%)
Sex	
Female	32 (56%)
Male	25 (44%)
pT size	
≤4 cm	22 (39%)
>4 cm	32 (56%)
Unknown	3 (5%)
pT stage	
T1	2 (4%)
T2	0 (0%)
T3	19 (32%)
T4	34 (60%)
Tx	2 (4%)
ETE	
No	9 (15%)
Yes	46 (81%)
Microscopic	12 (21%)
Gross	34 (60%)
Unknown	2 (4%)
Margins	
Negative	25 (44%)
Positive/close	30 (52%)
Unknown	2 (4%)
pN stage	
N0/Nx	25 (44%)
N1a	8 (14%)
N1b	23 (40%)
Unknown	1 (2%)
M stage	
M0	27 (47%)
M1	30 (53%)
Stage	
1	3 (5%)
2	0 (0%)
3	5 (9%)
4	47 (82%)
Unknown	2 (4%)
Tm grade	
PDTC	35 (61%)
WDTC	22 (39%)

Abbreviations: ETE, extrathyroid extension; pNx, clinically negative neck.

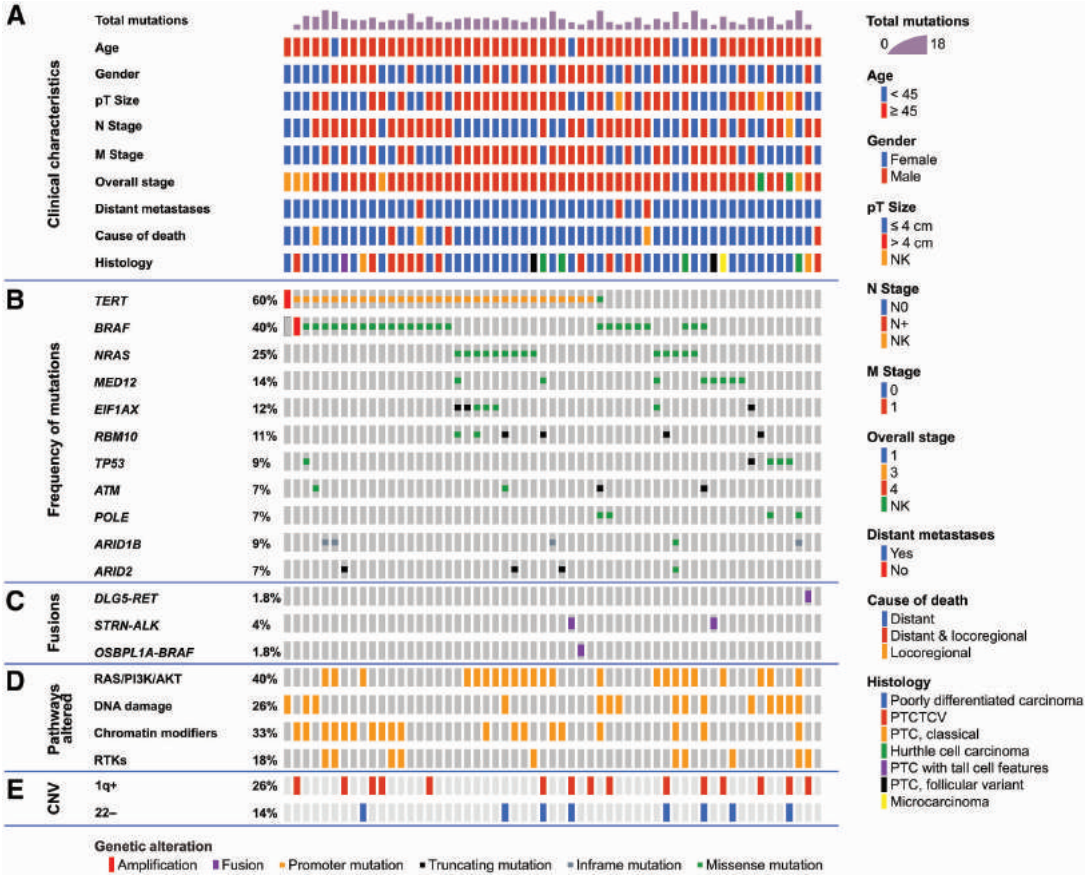


Figure 1 | Genomic landscape of fatal NAT cancer found in 410 genes in MSKCC IMPACT. Clinicopathological characteristics (**A**) included age, gender, tumor size, N stage, M stage, overall stage, distant metastases, and histology. The most frequent genes mutated are shown in (**B**) with fusions shown in (**C**). The percentage of tumors with alterations in different pathways is shown in (**D**); the RAS/PI3K/AKT pathway includes *NRAS*, *HRAS*, *PIK3CA*, *PTEN*, *PIK3C2G*, *PIK3CG*, *AKT1*, *AKT3*, *TSC2*, and *MTOR*; Chromatin modifier genes, including *KMT2A*, *KMT2C*, *KMT2D*, *KDM6A*, *PRDM1*, *BCOR*, *NCOA3*, *HIST1H3H*, *HIST1H1C*, *ARID1B*, *ARID2*, *SMARCB1*, *PBRM1*, *ATRX*, *CREBBP*. Alterations in DNA damage control included mutations in *TP53*, *RB1*, *MSK2*, *CHEK2*, and *POLE*. Alterations in RTKs included mutations in *PDGFRA*, *PDGFRB*, *FGFR3*, *ERBB3*, *MET*, *EGFR*, *TSHR*, *FGF3*, *TGFBR1*, *IFNGR1*. The percentage of tumors with gain of chromosome 1q and loss of chromosome 22 are shown in (**E**).

BRAFV600E mutations were present in 40%, and mutations in *NRAS* and *HRAS* occurred in 25% and 4%, respectively. *RAS* mutations were mutually exclusive with *BRAF* and gene fusions (Fig. 1). Mutations in the eukaryotic translation initiation factor *EIF1AX,* reported in 1% of PTCs (2), occurred in 12% of FNAT (Fig. 1 and Supplementary Fig. S1A), and were strongly associated with *RAS* (P< 0.001, Supplementary Fig. S1B). *EIF1AX* mutations clustered in two regions: the N-terminal domain, as also observed in uveal melanomas (10), or at a unique splice acceptor site between exons 5 and 6 (p.A113splice). This splice site is unique to thyroid cancer and also occurs with high frequency in ATC. It results in a 12-amino acid in-frame deletion (1).

Novel genes altered in fatal NAT cancer. Mutations of the gene *MED12* were seen in 14% of patients, and all *MED12* mutations were at the same site, resulting in an MED12-G44C substitution, consistent with a gain- or change-of-function (Fig. 2A). These were mutually exclusive to tumors with a *TERT* promoter mutation (P=0.04) and were also mutually exclusive to *BRAF* (P=0.011; Fig. 2B). The overall survival of patients with MED12 mutations were similar to those with wild-type MED12 (Fig. 2C).

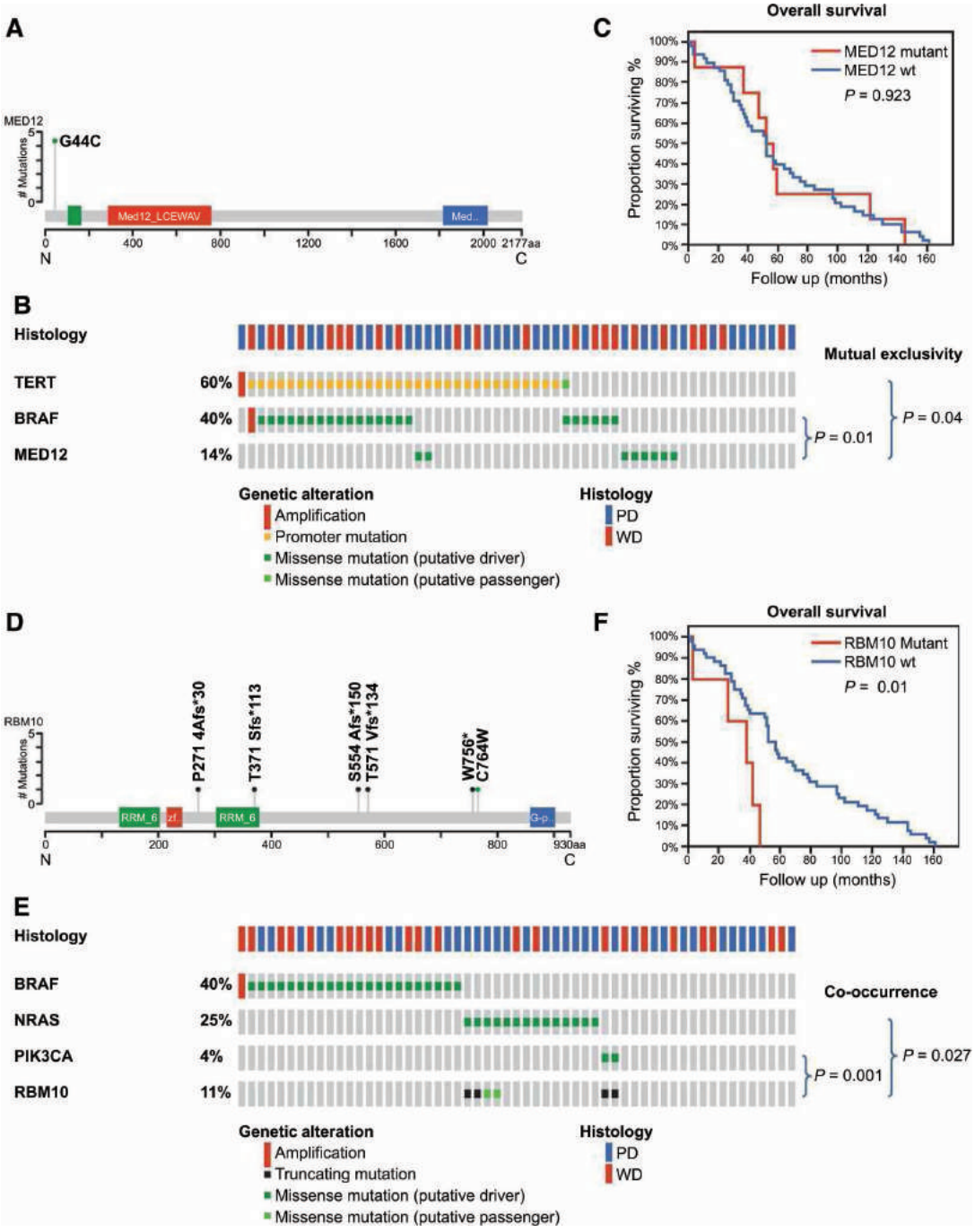


Figure 2 | Mutations in MED12 and RBM10 in fatal NAT cancer.

Mutation of the gene *RBM10* were seen in 11% of patients (Fig. 2D). *RBM10* mutations were of two types: four truncating mutations and two missense mutations. These were mutually exclusive to tumors with a *BRAF* mutation (P =0.037) but showed co-occurrence with *NRAS* (P= 0.027) and *PIK3CA* (P= 0.001; Fig. 2E). The overall

survival of patients with *RBM10* mutations was significantly poorer to those with wild-type RBM10 (P=0.01, Fig. 2F).

TP53, ATM, RB1, POLE mutations. We found mutations in *TP53* (9%), *ATM* (7%), and *RB1* (1.8%) at a higher prevalence than for PTCs in the TCGA (ref. 2; Supplementary Fig. S2A). Mutations in the *POLE* gene were seen in 4/57 (7%) of patients.

PI3K/AKT/mTOR pathway alterations. Mutations of genes encoding members of the PI3K/AKT/mTOR pathway were seen in 2557 (44%) of patients. Mutations occurred in *PIK3CA* (4%), *PTEN* (1.8%), *PIK3C2G* (5%), *PIK3CD* (1.8%), *PIK3CG* (1.8%), *PIK3R2* (1.8%), *AKT3* (1.8%), *TSC2* (1.8%), and *RPS6KA4* (1.8%). These mutations tend to be mutually exclusive to one another (Supplementary Fig. S2B).

Epigenetic gene alterations. Genes encoding components of the SWI/SNF chromatin remodeling complex were mutated in 9/57 (16%) of patients (Supplementary Fig. S2C). Mutations in *ARID1B* (9%), *ARID2* (7%), *SMARCB1* (4%), and *PBRM1* (1.8%) genes were identified. These mutations tended to be mutually exclusive, indicating that alteration in only one of these genes is sufficient to alter function. It was reported previously that individual SWI/SNF chromatin remodeling complexes can contain, for example, either ARID1A or ARID1B but not both. Namely, the combined absence of ARID1A and ARID1B destabilizes SWI/SNF complexes and results in dissociation of subunits which eventually leads to synthetic lethality (11).

Mutations of the histone methyltransferases (HMT) *KMT2A* (1.8%), *KMT2C* (4%-one amplification, one mutation), *KMT2D* (4%), *KDM6A* (1.8%) and *PRDM1* (7%; one mutation and three amplifications) were found in 10/57 (18%) of tumors (Supplementary Fig. S2C). Additional mutations in other chromatin remodeling and epigenetic regulators were also seen, including histone acetyltransferase *CREBBP* (1.8%) and *BCOR* (4%).

Other gene alterations. Other genes were mutated in a small number of patients. Mutations of other receptor tyrosine kinases (RTK) such as *PDGFRA* (1.8%), *PDGFRB* (1.8%), *FGFR3* (4%), *ERBB3* (1.8%) and *MET* (1.8%) were identified (Supplementary Fig. S2D). Mutations in *NOTCH2* (5%) and *NOTCH3* (1.8%) occurred in 4/57 (7%) of patients. There were infrequent mutations in *FLT3* (*VEGFR3*; 1.8%), *GNAQ* (1.8%), *GNAS* (4%), *KDR* (1.8%), *ASXL1* (1.8%), *DNMT1* (1.8%), *DNMT3A* (4%).

Gene fusions

Of the 57 patients, four had a gene fusion identified; one in a patient with PDTC and three in patients with WDTC (Fig. 1).

DLG5–RET (*DLG5*: 10q23;*RET*: 10q11.2) was identified in one WDTC patient with classical PTC. This is a balanced rearrangement involving *DLG5* exons 1–13, including the N-terminal coiled-coil domains and *RET* exons 12 and the rest of the downstream exons, which involves the entire *RET* kinase domain. This patient had no other mutations.

STRN–ALK (*STRN*: 2p22.2;*ALK*: 2p23) was identified in one WDTC(follicular variant of PTC) and one patient with PDTC. This is an 8 Mb deletion between *STRN* and *ALK* leading to a fusion between *STRN* exons 1–3 and *ALK* exons 20–29, which involves the entire *ALK* kinase domain. One patient also had a *TERT* promoter mutation, and the other patient had a *MED12* mutation.

OSBPL1A–BRAF (*OSBPL1A*: 18q11.1;*BRAF*: 7q34) was identified in one patient with WDTC with tall cell variant of PTC. This is a balanced rearrangement t(7;18) (q34;q18) involving *OSBPL1A* exons 1–16 and *BRAF* exons 10 and the rest of the downstream exons, including the entire *BRAF* kinase domain (AA 457-714). This patient also had a *TERT* promoter mutation.

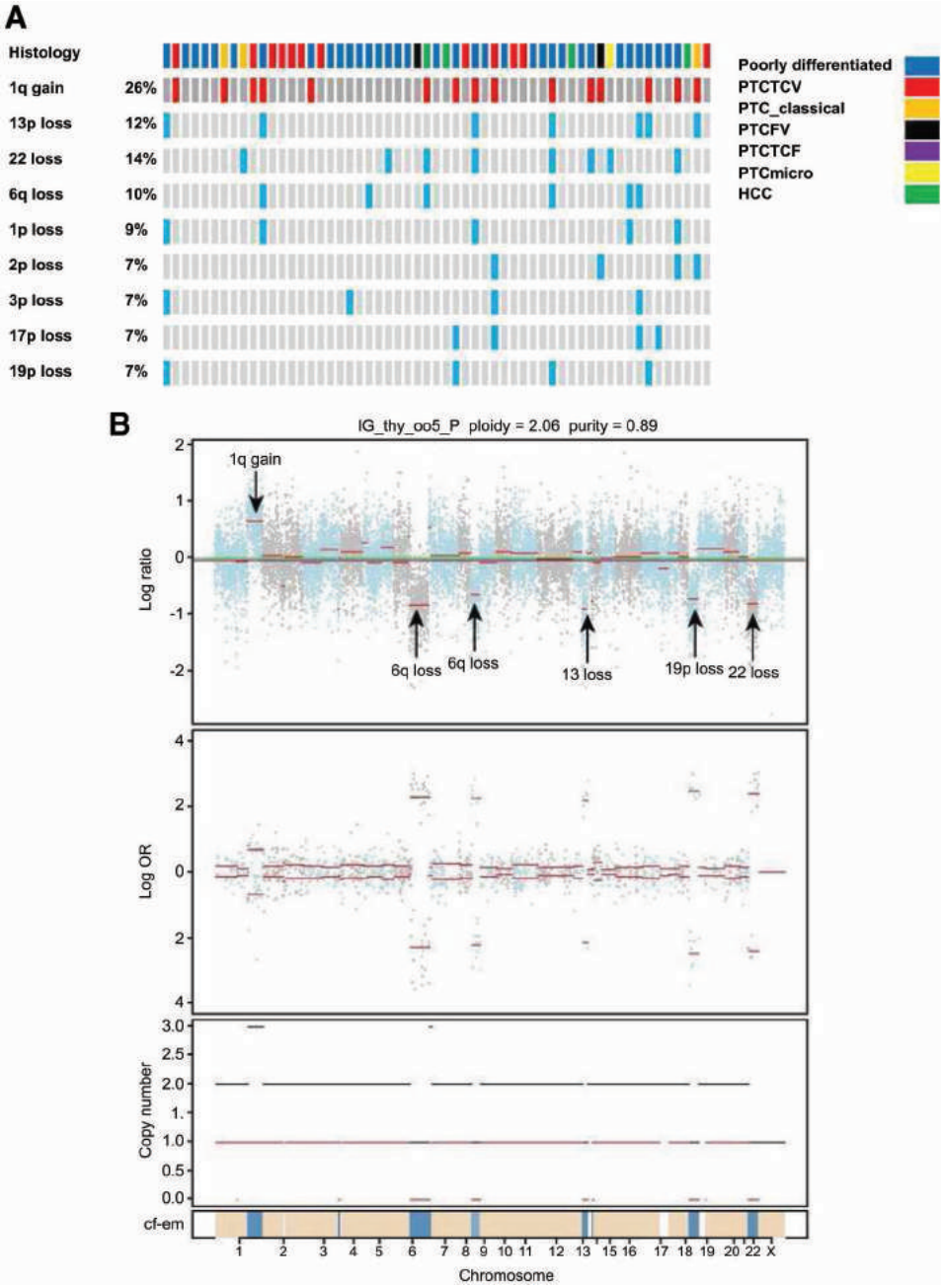


Figure 3 | Most frequent CNAs for each patient are shown in **A. B**, shows an example of copy-number alterations using FACETS in tumor IG_thy_005 that has 1q gain, 6q loss, 9p loss, partial 13 loss, 19p loss, and 22 loss.

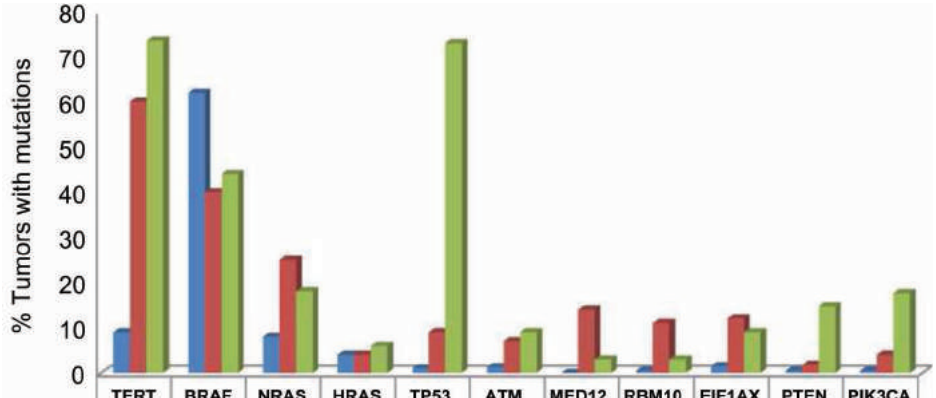
Copy-number alterations

Of the 57 tumors, there were several arm level alterations identified (Fig. 1). The most frequent copy-number alterations (CNAs) are shown in Fig. 3A. Arm level gains were identified in chromosome 1q in 15 (26%) patients. Arm level losses were identified in chromosomes 22 (14%), 13p (12%), 6q (10%), 1p (9%), 2p (7%), 3p (7%),

17p (7%), and 19p (7%). Example of CNAs in one tumor are shown in Fig. 3B; tumor IG_thy_005 has 1q gain, 6q loss, 9p loss, partial 13 loss, 19p loss and 22 loss. The different components of the FACETS plot are: The top figure shows the GC corrected normalized log-ratio of tumor to normal read depths at a set of SNP loci; the second figure is the log odds ratio from cross-tabulating the tumor and normal reads into ref and alt alleles for loci that are heterozygous in the germline; the third figure is the total (black) and minor (red) integer copy-number assignment for the segments and the final band shows the cellular fractions where dark blue represents one with lighter shades representing lower numbers and beige represents no copy-number change.

Pathways altered and mutational comparison with ATC and PTC

Overall, pathways altered in FNAT included the RAS/PI3K/AKT/MTOR pathway in 40% of patients, DNA damage pathway in 26% patients, chromatin modifying pathways in 33%, and alterations in RTKs in 18% of patients (Fig. 1). Figure 4 shows the most commonly mutated genes in FNAT compared with well-differentiated PTCs (TCGA) and ATC. Compared with WDTC reported in the TCGA, the prevalence of mutations of *TERT* (60% vs. 9%), *MED12* (14% vs. 0%), and *RBM10* (11% vs. 0.5%), are higher in FNAT indicating the importance of these genes in tumor virulence. Compared with ATC, FNAT showed a similar MAPK alteration profile with similar frequencies of mutations in *BRAF* (40%), *NRAS* (25%), and *HRAS* (4%) genes (1). Mutations in thyroid-stimulating hormone receptor gene (*TSHR*) were also comparable to 6% of ATC (1). Mutations in the eukaryotic translation initiation factor 1A, X-linked (*EIF1AX*), occurred in 12% of our FNAT which was comparable with 9% in our report on ATC (1) and higher than the 1% observed in PTCs (2).We have already reported before a significant association between *EIF1AX* and *RAS* mutation, suggesting that this may predict for more aggressive behavior (1). In contrast, mutations of PI3K/AKT/mTOR signaling were uncommon, occurring at a frequency more comparable to PTC (2). Mutations in tumor-suppressor genes *TP53* and *RB1* were much less common than in ATC. However, mutated *ATM* showed a similar rate of mutation in more aggressive tumors: 1.3% PTC (2) versus 7% FNAT versus 9% ATC (1). Mutations of the *POLE* gene were seen in 7% (4/57) of patients with FNAT. DNA polymerase epsilon catalytic subunit (*POLE*) gene mutations affect the active site of the exonuclease domain of DNA polymerase. This mutation has been described in familial forms of colorectal adenomas and cancers of the colon, pancreas, ovaries and small intestine (c.1373A>T; ref. 12) and in familial cutaneous melanoma (c.1041G>T; ref. 13).



	TERT	BRAF	NRAS	HRAS	TP53	ATM	MED12	RBM10	EIF1AX	PTEN	PIK3CA
TCGA %	9	62	8	4	1	1.3	0	0.5	1.5	0.5	0.5
FNAT %	60	40	25	4	9	7	14	11	12	1.8	4
ATC %	73.5	44	18	6	73	9	3	3	9	14.7	17.6

Figure 4 | Comparison of the most commonly mutated genes in WDTC (TCGA), fatal cases of NAT cancer, and ATC.

FNAT comprise both WDTC and PDTC. A comparison of fatal WDTC with nonfatal WDTC using the TCGA cohort is shown in Supplementary Fig. S3A. From this, we can conclude that the prevalence of mutations of *TERT* (60% vs. 9%), *MED12* (13% vs. 0%), *RBM10* (4% vs. 0.5%), and *PIK3CA* (4% vs. 0.5%) are higher in fatal forms of WDTC. We have also shown the comparison of fatal PDTC to nonfatal PDTC using nonfatal PDTC identified from the cohort in Landa and colleagues (1). This is shown in Supplementary Fig. S3B. From this we can conclude that the prevalence of mutations of *TERT* (60% vs. 21%), *MED12* (15% vs. 0%), *RBM10* (12% vs. 0%) are also higher in fatal forms of PDTC. In addition, prevalence of other genes is higher in fatal forms of PDTC. These included *BRAF* (29%vs. 4%), *HRAS* (6%vs. 1.8%), *TP53* (15%vs. 9%), *ATM* (12% vs. 0%), and *EIF1AX* (21% vs. 4%).

Molecular profile of FNAT carcinomas according to their various histotypes

The molecular alterations categorized by histology are shown in Fig. 5. Of the 13 patients with tall cell variant or tall cell features, nine had both a *TERT* promoter mutations and *BRAF* mutation, indicating the importance of this combination in this histology. Of two patients with PTC classical type, one patient had a *TERT* promoter mutation with a *BRAF* mutation, and the other patient harbored the *DLG5–RET* fusion gene. There was one patient with a PTC microcarcinoma, and this patient had an *MED12* mutation. There were four patients with Hurthle cell cancer; one patient had *TERT*, *MED12*, *RBM10* and *PIK3CA* mutations, one patient had *TERT* and *ARID2* mutations, one patient had *NRAS* mutation, and one patient had *ARID1B*, *POLE*, *TSHR*, *FGFR3*, *TSC2*, *PDGFRB* and *ERBB3* mutations.

Of patients with PDTC, 60% had a *TERT* promoter mutation either with a *BRAF* mutation or a NRAS mutation. Five patients with *TERT*/*NRAS* mutations also harbored an *EIF1AX* mutation. All *EIF1AX* mutations occurred in the patients with PDTC usually in combination with *NRAS* mutations. Of the patients who did not have a *TERT* promoter mutation, mutations were observed in *MED12*, *RBM10*, *TP53*, and *ATM* among others.

Discussion

In this study, we report a mutational assessment and clinicopathological features of 57 fatal cases of NAT (FNAT) cancer and we examine our results in the context of PTC TCGA study (2), and in the context of deep-sequencing studies of ATC we reported previously (1).

TERT promoter mutations showed the highest prevalence of mutation occurring in 60% of FNAT patients. These occurred with equivalent frequencies in both PDTC (60%) and WDTC (60%) patients. Promoter mutations occurred in the two usual hotspot positions (14). The 60% mutation rate in FNAT is far higher than the 9% of PTCs from TCGA (2) and comparable with 73% of ATC (1). This stepwise increase in the frequency of *TERT* promoter mutations as thyroid differentiation decreases is consistent with our previous reports (1, 15) and reports from other studies (16). *TERT* mutations co-occurred with *BRAF* or *RAS*, which enhance the negative prognostic impact of *TERT* promoter mutations (17). In contrast, *TERT* promoter mutations in FNAT were mutually exclusive with *TP53* mutations (P=0.08) and with *MED12* mutations (P=0.04), consistent with alternate pathways toward progression to FNAT.

Compared to ATC, FNAT also showed a similar MAPK alteration profile (*NRAS*, *HRAS*, and *BRAF*) and a similar incidence of mutations in the eukaryotic translation initiation factor 1A, X– linked (*EIF1AX*; ref. 18). In contrast, mutations of PI3K/AKT/ mTOR signaling were uncommon, occurring at a frequency more comparable to PTC (2). In addition, mutations in tumor suppressor genes *TP53* and *RB1* were much less common than in ATC, supporting the notion that mutations in TP53 are infrequent in all histologic types of thyroid cancer with the exception of ATC (1).

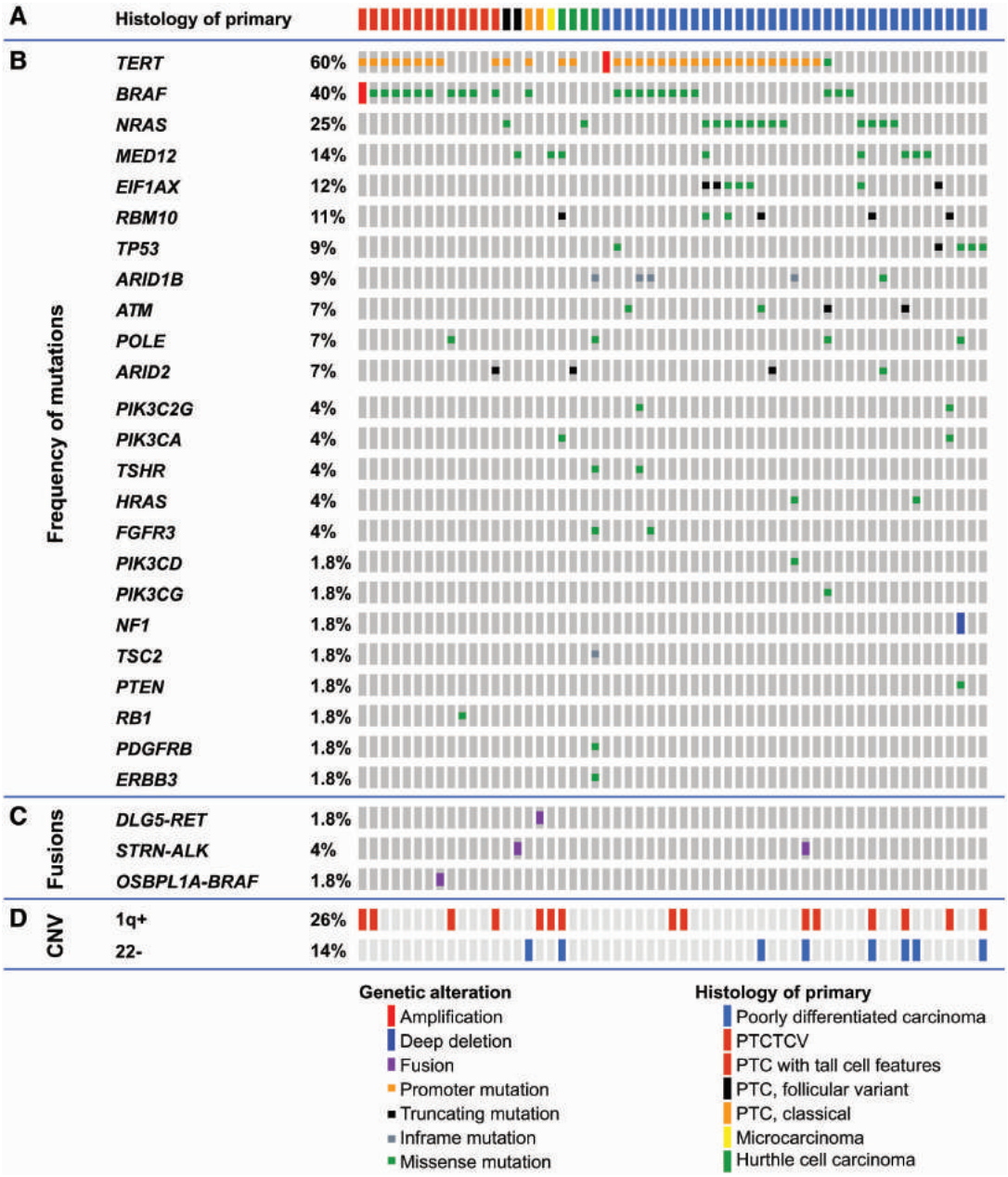


Figure 5 | Molecular alterations in FNAT stratified by histology.

We identified chromosomal rearrangements in only a small percentage of FNAT (4/57; 7%) and all involved the entire kinase domain of the fusion partner. Importantly, two of the three fusion genes identified have never been reported before (*DLG5–RET* and *OSBPL1A–BRAF*). *DLG5–RET* fusion involved the entire *RET* kinase domain and therefore *DLG5* may lead to constitutive activation of *RET* kinase. Disc large homolog 5 (*DLG5*) gene is located in a region that undergoes substantial recombination and has a possible role in inflammatory bowel and Crohns disease (19), in cell division, proliferation, cell migration and invasion (20); however, it has

not been reported as a *RET* partner in chromosomal rearrangements. As with other *RET* fusion partners, the *DLG5* gene has a coiled-coil domain which acts as the dimerization domain for the *RET* gene. *OSBPL1A–BRAF* is a balanced rearrangement that involves the entire *BRAF* kinase domain. In this novel fusion, *OSBPL1A* may lead to constitutive activation of *BRAF* kinase. *OSBPL1A* (Oxysterol-binding protein–related protein 1, which acts as an intracellular lipid receptor which is a member of the oxysterol- binding protein family) was shown to have differential expression of isoforms in several cancer types as a result of alternative transcription start site (in colorectal, lung, bladder, liver, prostate, gastric, and brain cancer; ref. 21). The other fusion identified was *STRN–ALK*. This involves the entire *ALK* kinase domain, which leads to constitutive activation of *ALK* kinase via dimerization mediated by the coiled-coil domain of *STRN* (22). This rare rearrangement has been reported in thyroid cancer before (2) as well as renal cell carcinoma (23) and colorectal adenocarcinoma (24). Patients with this fusion have shown significant initial clinical response to the ALK inhibitors crizotinib and TAE864.

Several CNAs were identified with the most common being gain of chromosome 1q and also loss of chromosome 22. Chromosome 1q gain was present in 15 patients with FNAT (26%), which is higher than 14.8% (2) and 16% (25) reported in PTC. In PTC, 1q gain has been reported in more aggressive tumors (26) and associated with significantly higher MACIS scores, risk profiles and PTC tumor stage (2) as well as distant metastases (25). In PDTC, 1q gains were among the most common arm level CNA (1) and patients with PDTC with 1q gains had worse survival rates (1). The high incidence of 1q gain that we observe is in keeping with these findings. Arm level losses in chromosome 22 were present in eight patients with FNAT (14%). This corresponds with previous reports on PTC and PDTC where 22q loss was reported (1, 2, 25). Loss of 22q region includes tumor-suppressor genes *NF2* and *CHEK2* (25). *NF2* loss promotes *RAS* induced tumorigenesis (27), which is consistent with strong association between 22q loss and *RAS*-mutated PDTC (1). Therefore, our report of 1q gain and 22 loss in FNAT is consistent with previous reports of these CNA in aggressive thyroid tumors.

Our study identified a remarkably high prevalence of mutations in two genes, *MED12* and *RBM10*, suggesting a role of these genes in tumor virulence. When we carried out a comparison of fatal forms of WDTC and PDTC to nonfatal forms of WDTC and PDTC, these two genes had a higher mutation prevalence indicating their importance in both WDTC and PDTC tumor virulence. *MED12* (Mediator of RNA polymerase II transcription subunit 12 homolog) is located on X chromosome and encodes for a subunit of the macromolecular complex known as Mediator. Mediator complex consists of the core Mediator and the kinase module and initiates DNA transcription by interacting with RNA polymerase II (RNA Pol II; ref. 28). Because *MED12* plays an essential role in the assembly and activation of the kinase module (29, 30) mutations in *MED12* can lead to loss or gain of kinase activity. The latter can act as a promoter or suppressor of tumorigenesis, depending on biologic function that the kinase module carries out in the particular tissue (31, 32).

MED12 has recently been included as a cancer driver gene in a recent large-scale genomic analyses (33, 34), reflecting its growing importance. In our study of FNAT, *MED12* mutation clustered in a hotspot region in the N terminal region of exon 2 and this clustering suggests a specific change of function. This is consistent with the vast majority of reports where missense mutations of *MED12* clustered in a hotspot region within exon 2 (31). Mutation of *MED12* has been reported to alter highly conserved amino acids residues (L36, Q43, and G44) in exon 2 (35) that points to a possible gain or change of function. Exon 2 mutations were initially found in uterine leiomyomas (UL; ref.35) and were the first *MED12* mutations reported in human tumors which implicated the role for disrupted Mediator associated CDK8 kinase activity in tumorigenesis. Since then, *MED12* mutations have been reported in typical UL (up to 86%), breast fibroadenomas (59%–67%) and phyllodes tumors (80%–88%; ref. 31). Comparative expression profiling and gene set enrichment analyses from mutant and wt *MED12* reveal TGF-beta s ignaling and Wnt/beta-cateni n signaling in mutant UL (31). Further research is needed to determine the impact of *MED12* exon 2 mutations on the estrogen signaling pathway

and its possible dysregulation during tumorigenesis. Exon 2 mutations are recurrent albeit less frequent in malignant uterine leiomyosarcomas (4%–30%), chronic lymphocytic leukemias (5%) and colorectal cancers (0.5%; ref. 31). *MED12* mutations have also been reported outside of exon 2 in 5% of prostate cancers and may act through disruption of CDK8 kinase with subsequent transcriptional dysregulation of p53 and androgen signaling (36, 37). In our study, all *MED12* mutations were recurrent mutation in a single codon resulting in a MED12–G44C substitution. We anticipate that the *MED12* mutations in our patients may represent gain- or change-of-function. However, given the complexity of the Mediator complex and the variability in either gain- or loss- of-function depending on tumor type, more research is required to properly explore the true function of the *MED12* mutation that we have identified.

In addition to *MED12* mutations, we also found mutations in the RNA Binding Motif Protein 10 (*RBM10*) in 11% of patients with FNAT. RBM10 is an RNA-binding protein that participates in alternative pre-mRNA splicing. Splicing has a direct role in regulation of gene expression and maintaining the homeostasis of cellular processes. Indeed, there has been growing evidence of the involvement of mutated splicing factors in tumor progression (38). Mutation of splicing factors can impair expression of genes crucial for maintaining homeostasis of cell growth, and therefore represents a novel mechanism, which may promote growth advantage and tumorigenesis of select clonal populations. Mutations of genes encoding splicing factors have been most commonly reported in hematologic malignancies [myelodysplastic syndromes (MDSs), acute myelogenous leukemia, and chronic lymphocytic leukemia], and less frequently in several solid tumors (38). Most frequent mutations occur in *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2* and are generally mutually exclusive (38). With regards to *RBM10*, mutations have been reported in lung adenocarcinomas (39, 40), where it acts as an alternative splicing regulator (41) modulating the product of *NUMB*, a NOTCH pathway regulator gene (42) critical for progression of lung adenocarcinomas. Studies point to loss of tumor suppressor properties of wild-type *RBM10* and oncogenic function of mutated *RBM10* as possible mechanisms for causing uncontrolled growth (40). *RBM10* knockdown (*RBM10KD*) in human cancer cells enhanced tumor growth of xenografts in nude mice with similar results in lung adenocarcinoma cells expressing an RBM10 valine to glutamic acid (V354E) substitution (40). In addition to missense mutations, truncation mutations also occur in *RBM10*. RBM10 truncated mutants lacking the C-terminal Zn-finger and glycin patch are basically non-functional; moreover, the shortest variants appear to exert a dominant-negative effect (40). In our study, *RBM10* mutations were of two types: four truncating mutations and two missense mutations. We anticipate that the *RBM10* mutations in our patients may represent loss of tumor suppressor function. Furthermore, our study showed statistically significant co-occurrence of mutation in *RBM10* with *NRAS* and *PI3KCA* and also a mutual exclusivity with *BRAF* mutations. This suggests mutual independence in oncogenic potential of *RBM10* regulated proteins and BRAF signaling. Importantly, patients who had *RBM10* mutations had a significantly poorer survival compared to patients who did not have these mutations. This suggests tumors harboring *RBM10* mutations are biologically more virulent.

Our study has identified several genetic alterations that may have therapeutic implication. There is a great interest to specifically target mutated *TERT* promoter, and our findings suggest aggressive thyroid cancer with these mutations would be an ideal cancer to treat. In addition, new insights into *TERT* genetics and biology may also offer a potential for personalized immunotherapy (43). The rare *STRN–ALK* rearrangement can be targeted with ALK inhibitors, crizotinib and TAE864, as previously mentioned. The central role of *MED12* in the proper function and assembly of the Mediator kinase module makes *MED12* an attractive therapeutic target. So far efforts in targeted therapy have mostly been directed toward CDK8 kinase; for example, Sorafenib as a multi-tyrosine kinase inhibitor/CDK8 inhibitor and Senexin A as a novel CDK8/19 inhibitor (31). An important obstacle in targeting Mediator kinase module may be its versatile role in both repression and activation of transcription depending on the context (31, 44) and consequent impact on oncogenic or tumor suppressor

signaling. Mutated *RBM10* may also represent a novel therapeutic strategy at the level of transcription. Splicing factor inhibitors have already been tested in clinical trials but not in patients with splicing factor mutations (38). A phase I trial of E7107, a spliceosome inhibitor, have been conducted in patients with advanced solid tumors unresponsive to standard therapies (45) and showed promising results. It is possible that splicing factor inhibitors may also be useful in patients that harbor *RBM10* mutations.

In conclusion, we report the mutational profile of the largest series of fatal NAT that has not been reported before. We have identified *TERT* promoter mutations in a very high percentage of tumors, indicating its importance in thyroid cancer virulence. We report a high incidence of chromosome 1q gain that highlights its importance in tumor aggressiveness. We have identified two novel fusion genes *DLG5–RET* and *OSBPL1A–BRAF*. Finally, we report on many novel genes not previously reported in differentiated thyroid cancer including *MED12* and *RBM10*, suggesting a role for these novel genes in tumor virulence. These new data will clarify the genetic basis of the most virulent forms of thyroid cancer, and will therefore help focus future therapeutic directions.

Supplementary data for this study are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Chapter 10

Discussion, Conclusions, Future directions

Discussion

Our studies show that poorly differentiated thyroid cancer (PDTC) is a rare type of thyroid cancer. During 1986-2009 period 3493 previously untreated patients with thyroid cancer underwent surgery at MSKCC, and of these only 91 patients (2.6%) were diagnosed to have PDTC using the criteria of proliferative grading. This is in agreement with reported incidence of PDTC in North America which ranges from 2-3% of all thyroid malignancies (1). Our research showed that PDTC is a highly significant entity, representing the most common cause of death from non-anaplastic thyroid cancer (57% of fatal non-anaplastic cases were PDTC). Our PDTC patients were older (median age 59 years) than generally reported for thyroid cancer (median age 51 years) (2) and had higher male to female ratio (1:1.6 vs. 1:3 in reports on thyroid cancer) (2). Older age and a higher proportion of male gender in our PDTC patients indicate aggressive tumor biology since these demographic features have been reported as adverse prognostic factors in thyroid cancer (3,4). Aggressive tumor biology of PDTC is further reflected in its clinicopathological characteristics. More than half of our patients with PDTC presented with gross extrathyroid extension, with high incidence of regional lymph node metastasis and distant metastasis at presentation and during the course of follow-up (41% of our PDTC patients had distant disease vs. less than 10% reported for DTC (5)). We separately studied a cohort of PDTC patients with gross extrathyroid extension (ETE) at presentation (cohort amenable to surgical treatment: Stage T4a). This aggressive subgroup of PDTC with ETE were considerably older (median age 70 years) compared to the rest of PDTC patients and with gender gap almost disappearing (female to male ratio close to 1:1). This confirms the development of a more aggressive PDTC biology in older and male patients. Compared to reports on differentiated thyroid cancer (DTC) with gross ETE, our PDTC cohort with gross ETE showed higher propensity to invade more posteriorly located structures (e.g. recurrent laryngeal nerve or esophagus) which is a reflection of their more aggressive nature and tendency to have larger tumors.

Initial treatment strategy of surgical clearance of all gross disease together with neck dissection, in case of clinical or radiological presence of suspicious lymph nodes, was successful in the majority of patients (92% of PDTC patients without and 74% of PDTC patients with gross ETE had no gross residual disease). The initial therapy resulted in satisfactory 5 year locoregional control in 81% of PDTC patients without and 56% of PDTC patients with gross ETE. The majority of our patients (77%) received adjuvant therapy with radioiodine (RAI) and/or external beam radiation therapy (EBRT) due to the potential risk of recurrent disease. However it is difficult to draw conclusions on the efficacy of adjuvant treatment on disease control due to small number of patients. Radioactive Iodine was the most common adjuvant therapy in our PDTC patients. Although 70% of patients with M1 disease were RAI-avid, this treatment was not therapeutic and distant disease was persistent in all patients after adjuvant RAI. This can be explained on the basis of tumor heterogeneity with mixed presence of well differentiated RAI avid component and poorly differentiated areas that don't respond to RAI. EBRT was employed in a small number of patients due either gross residual disease, positive margins or advanced age and aggressive tumor histology. Although EBRT benefits on local control of PDTC are inconclusive, it may be considered in high risk settings due to a low toxicity profile with IMRT. The 5 year disease specific survival in our patients was low (66% in all PDTC and 49% in PDTC with ETE). PDTC patients who died of the disease compared to those who survived were significantly older, presented with larger tumors, local invasion, higher stage and/or distant metastases. Multivariate analysis revealed pT4a and M1 as significant predictors of worse outcome. This is in agreement with our findings of a strong relationship between stage T4a disease and development of distant metastases (59% of T4a patients had distant metastases). Distant disease represented the cause of death in the majority of our patients. Since traditional modes of treatment achieve high rates of locoregional but not distant control, we emphasize the need for the development of systemic targeted therapies in order to control distant disease progression and to improve overall outcomes.

Our data show that MSKCC-PDTC diagnostic criteria based on proliferative grading of mitosis and necrosis (6) define a biologically more homogenous group of aggressive tumors. When MSKCC-PDTC criteria were used to differentiate between PDTC and other histotypes in fatal non-anaplastic follicular cell-derived thyroid cancer (non-ANA FCDC) patients, all patients with non-ANA FCDC who died of the disease had at least one of four aggressive features: gross ETE, extensive vascular invasion (VI), PDTC diagnosis in the primary tumor or the neck or distant metastasis (DM) at presentation. Therefore in fatal non-ANA FCDC, presence of at least one of these four features can signal the risk of disease related mortality and should therefore prompt more aggressive initial therapy and more close follow-up. However when we used Turin-PDTC criteria to differentiate between PDTC and other histotypes, some tumors were labeled as a variant of DTC with no other risk factors. Turin-PDTC criteria may therefore result in tumor diagnosis that escapes higher risk stratification and may mislead the clinician into less aggressive initial treatment and less aggressive follow-up.

In the studies from 1990s from Japan, immediate cause of death from fatal DTC was solely uncontrollable locoregional disease in 29% -35% of patients (7,8). In our study on fatal non-ANA FCDC <10% of patients died of locoregional disease while in our study on PDTC 18% of disease-specific deaths were due to locoregional disease. In the most invasive cohort, characterized by gross ETE, 21% of disease-specific deaths were due to locoregional disease. When compared to previous reports from the litereature, improvement in locoregional control in our patients may reflect more comprehensive initial surgery and successful gross disease clearance.

Thyroglobulin (Tg) has been a well established follow-up marker for DTC. Patients with GAMES stratification (9) low-risk PTC (< 45 years with pT1) and undetectable postoperative Tg have low risk of recurrence. Here we report that a select group of patients with GAMES (9) intermediate-risk PTC (≥ 45 years but with pT1T2 and negative neck) and undetectable Tg also have low risk of recurrence. Therefore with proper selection and an adequate follow-up, those PTC patients with low risk of recurrence can be safely managed without adjuvant RAI. In case of PDTC, most tumors have lost well-differentiated features and the ability to produce Tg. However some PDTC retain the ability to produce Tg, as a result of heterogeneity with some components of the tumor showing focal areas of well differentiated tumor. Due to the lack of reports on Tg in PDTC, it is not clear if Tg has any predictive value on recurrence. Our PDTC patients with M0 and undetectable Tg after surgery and adjuvant RAI had significantly higher rates of 5 year recurrence-free survival (RFS) and regional recurrence-free survival (RRFS) than patients with detectable Tg. However, our results on PDTC and Tg levels must be interpreted cautiously in light of tumor heterogeneity in PDTC. 8% of our PDTC patients with undetectable Tg had distant recurrence. This shows that less differentiated tumor component produces no Tg and therefore can be responsible for persistent disease and recurrence despite undetectable Tg. Furthermore, PDTC patients in out study were M0 at presentation and it is likely that PDTC had a higher representation of better differentiated component than more aggressive types of PDTC. We conclude that undetectable Tg represents a marker or initial disease clearance and therefore can predict low risk of recurrence in a select group of PTC and PDTC patients. However, patients with tumors that are higher on the progression spectrum, which are likely to contain less differentiated tumor components with no Tg production capability, may have persistent disease post surgery and can recur, despite initial undetectable Tg values. Therefore, they need to be more closely monitored by structural imaging studies during follow-up.

We also examined the genomic profile of PDTC in order to determine molecular markers of disease aggressiveness. Our study on *TERT* promoter mutations reports for the first time a high frequency of these mutations in follicular cell-derived thyroid cancer. The telomerase reverse transcriptase (TERT) has a highly specific function in preserving the telomere ends, preventing apoptosis and rendering tumors immortal. *TERT* promoter is the most important regulator of telomerase expression since it contains several binding sites for transcription factors (10). Mutations of *TERT* promoter generate a consensus-binding site (GGAA) for

E twenty-six (ETS) transcription factors which confer enhanced *TERT* promoter activity (11). High rates of *TERT* promoter mutations in thyroid cancer may be explained by terminally differentiated nature of thyroid cells, which show a very low mitotic rate postnatally (12). Indeed, it has been proposed that *TERT* activating mutations may be more common among cancers derived from cells with low self-renewal capacity which are thus likely to benefit from maintained telomerase lengthening (13). In contrast, rapidly renewing tissues already possess alternative mechanisms of telomerase lengthening (13) and therefore *TERT* activating mutations are less likely to confer clonal advantage. We found that *TERT* promoter mutations were significantly overrepresented in advanced types of thyroid cancer such as PDTC and ATC when compared to PTC. This is consistent with reports on other types of cancer, where *TERT* promoter mutations were also enriched in advanced stages; e.g. in metastatic melanomas (11) and adult primary glioblastomas (13). *TERT* promoter mutations may therefore serve as a novel biomarker of tumor progression. *TERT* C228T was a more common type of mutation than C250T and the two showed mutual exclusivity, which probably reflects sufficiency of each mutation in driving tumor progression. We also found significant co-occurrence of *TERT* promoter mutations with *BRAF* or *RAS* mutations in PDTC and ATC. This co-occurrence is likely associated with de novo consensus binding sites for ETS factors in *TERT* promoter as a result of either C228T or C250T mutation (11,14). Expression of ETS family of transcription factors is enhanced by MAPK output, driven by mutated *BRAF* or *RAS* (15). In turn *TERT* promoter could also extend the lifespan of *BRAF*-or *RAS*- driven clones with progressive accumulation of other genetic alterations and development of advanced disease. Besides *TERT* promoter mutations, alternative mechanisms of *TERT* activation have also been proposed. Aberrant *TERT* promoter methylation (16) or inactivating mutations in the *ATRX* gene, a Rad54-like ATP-driven DNA translocase, which cause telomere lengthening (17) represent alternative mechanisms of illegitimate *TERT* activation.

We used ultra deep next-generation sequencing (NGS) technology for MSK-IMPACT targeted sequencing of 341 cancer relevant genes in one of the largest cohorts of PDTC (84 patients) that were reported from a single tertiary care institution. We compared our results to those from ATC treated at our institution and sequenced concurrently by NGS and to PTC from TCGA study (18). Our results show a molecular level evidence of thyroid cancer progression whereby PDTC holds an intermediate position between DTC and ATC. The mutation burden increased significantly from PTC towards ATC, pointing to genomic instability and accumulation of mutations with thyroid cancer dedifferentiation. We also found that the aggressive clinical characteristics paralleled mutation accumulation; in PDTC the mutation burden was significantly associated with older age, larger tumors, distant metastasis and reduced overall survival. Accumulation of mutations therefore represents a molecular background of dedifferentiation process and acquisition of aggressive behavior. We found a wide variety of genomic alterations and at higher frequencies in advanced tumors compared to TCGA PTC (18); single nucleotide polymorphism, indels, structural variations like copy number alterations (CNA) and genetic fusions. PTC and more advanced tumors share the most common driver mutations; *BRAF* and *RAS*. However, *BRAF* mutations were less prevalent in advanced tumors while *RAS* mutations were more frequent in advanced tumors when compared to PTC. *BRAF* mutated PDTC showed significantly higher rates of regional nodal metastases and *RAS* mutated PDTC showed significantly higher rates of distant metastases, consistent with the clinical behavior of mutated *BRAF* and *RAS* in DTC (19). In addition, we found a strong association of PDTC-Turin tumors with *RAS* mutations and PDTC-MSKCC tumors with *BRAF* mutations. This may have important clinical implications when applying different diagnostic criteria due to different metastatic tropism of these mutations. Based on an expression pattern of a gene panel for BRAFV600E and *RAS* mutated tumors, BRAF-RAS score (BRS) has been reported previously (18). High MAPK transcriptional output is characteristic of BRAFV600E mutated tumors in PTC (18). This can be explained by the monomeric nature of BRAFV600E signaling, causing its unresponsiveness to the negative feedback by ERK, resulting in a high MAPK output. In *RAS* mutated tumors, negative feedback by ERK is successful in disruption of BRAF dimerization, resulting in an attenuated MAPK output (15,18). This clear demarcation of the feedback mechanism was reflected in gene

expression patterns in PDTC similar to report on TCGA PTC (18), however it was lost in ATC. Specifically, in our PDTC BRS correlated with their *BRAF or RAS* mutational status. However this correlation was largely lost in ATC, whereby both *BRAF* and *RAS* mutated ATC showed BRAF- like BRS. The latter suggests that a high MAPK output exist in ATC regardless of *BRAF or RAS* mutation status. Similarly thyroid differentiation score (TDS), that is based on an expression pattern of a gene panel involved in iodine metabolism and thyroid differentiation, showed comparable mRNA expression patterns between PTC and PDTC in relation to mutated drivers. ATC again showed significant suppression of a number of genes with loss of clear association between TDS gene expression and underlying driver mutations. Loss of BRS and TDS association to the underlying drivers in the most advanced thyroid carcinoma is in agreement with the progression model of carcinogenesis. While these associations were still maintained in PDTC similar to PTC, blurring of these associations in ATC can be attributed to the accumulation of additional genomic complexity such as higher frequencies in mutations in chromatin modifiers or proteins that activate parallel signaling pathways. In addition, high frequency of specific genomic alterations in advanced tumors further support the progression model of thyroid carcinogenesis. Activating mutations of *TERT* promoter also displayed a stepwise increase in mutation frequency along the spectrum of disease progression: they were found in 9% of PTC (20), 40% of PDTC and 73% of ATC. This is consistent with our previous findings of *TERT* mutations accumulation in advanced thyroid cancer by automated capillary sequencer technology. We also found that *TERT* promoter mutations were subclonal in PTC and clonal in PDTC and ATC, which speaks for clonal selection during tumor evolution and possible induction of cell immortalization in PDTC and ATC. We found significant association between *TERT* promoter mutations and *BRAF* or *RAS* mutations in PDTC and ATC, which is consistent with the mechanism of *TERT* promoter mutations generating de novo consensus motifs for the ETS family of transcription factors that are activated by MAPK signaling, which ultimately results in TERT overexpression. *TERT* promoter mutations can in turn introduce an immortalized phenotype in advanced tumors. In contrast with *TERT* mutations, *TP53* mutations were not frequent in our PDTC tumors, showing significantly lower prevalence when compared to ATC (8% vs. 73%). This is also in contrast with earlier reports, where *TP53* was the most frequently mutated gene in PDTC (27%) (21). In addition we found significantly less prevalence of mutations in genes encoding for members of PI3K/AKT/mTOR pathway in PDTC compared to ATC (11% vs. 39%). Therefore accumulation of mutations in *TERT* promoter, *TP53* and PI3K pathway genes from PDTC to ATC may represent key genetic events distinguishing between PDTC and ATC. In addition alterations in the functional programs not previously associated with thyroid cancer may also serve as delineation between PDTC and ATC; e.g. alterations in switch/sucrose non-fermentable (SWI/SNF) chromatin remodelling complexes and histone methyltransferases (HMTs) were significantly higher in ATC vs. PDTC we studied. Furthermore we analyzed copy number alterations (CNA) which showed distinctive distributions in PDTC and ATC. PDTC had greater frequency of 1p losses, whereas 8p and 17p losses, as well as 20q gains, were more common in ATC. Furthermore, 22q losses were strongly associated with *RAS* mutant PDTC. Loss of the 22q tumor suppressor gene *NF2* with concurrent *RAS* mutation leads to PDTC development in mice. *NF2* encodes for the protein merlin whose loss causes inactivation of the Hippo pathway and in turn YAP-TEAD–dependent transcriptional activation of oncogenic and wild type *RAS*, with enhancement of MAPK transcriptional output and promotion of transformation (18,22). Mutations of *EIF1AX*, which encodes for translation initiation factor, occurred more frequently in our advanced thyroid cancers compared to TCGA PTC (18). The mutations of EIF1AX cluster in specific N- and C-terminal residues. Mutation of unique splice acceptor site between exons 5 and 6 (p.A113splice) is specific for thyroid cancer and predicts for alternative usage of a cryptic splice acceptor within exon 6 which causes in-frame deletion of 12–amino acids. N-terminal and C-terminal mutations in yeast disrupt EIF1A function in regulating the conformation of the translation preinitiation complex and scanning for the AUG initiation codon (23). We found comparable frequencies of *EIF1AX* mutations in PDTC and ATC (10%), with strong co-occurrence of mutated *EIF1AX* and *RAS*, which was not present in PTC. The impact of this co-occurrence on thyroid carcinogenesis is

currently unclear. *EIF1AX* mutations are mutually exclusive with mutations in the PI3K/AKT/mTOR pathway which points to a possible overlap of functional gains. In terms of prognosis, *EIF1AX* mutations are predictive of worse survival in PDTC and may therefore represent a useful marker for risk stratification in these tumors.

We also performed ultra deep NGS technology for MSK-IMPACT targeted sequencing of recent 410 cancer relevant genes in fatal cases of non-anaplastic thyroid cancer (FNAT). We report the genomic profile and clinicopathological features of 57 FNAT patients (35 PDTC and 22 WDTC) in the context of PTC TCGA study (18) and our previously reported deep sequencing studies of ATC (20). *TERT* promoter mutations were the most prevalent type of mutations among FNAT (60% of PDTC and 60% of WDTC). The stepwise increase in prevalence of *TERT* promoter mutations between TCGA PTC vs. FNAT and ATC (9% vs. 60% and 73% respectively) correlates with decrease in thyroid differentiation and is consistent with our previous findings (20,24) and reports from the literature (25). The co-occurrence of *TERT* mutations with *BRAF* or *RAS* mutations in FNAT is consistent with our previous findings in advance thyroid cancers (20) and is in agreement with enhancement of the negative prognostic impact of mutated *TERT* promoter by simultaneous presence of *BRAF*/*RAS* mutations (26). On the contrary, mutual exclusivity of *TERT* promoter mutations with *TP53* mutations (p=0.08) and with *MED12* mutations (p=0.04) is consistent with alternate pathways of progression towards FNAT. Similar MAPK alteration profile (*BRAF*, *NRAS*, *HRAS*) and similar prevalence of *EIF1AX* mutations in FNAT and ATC (20) indicate importance of these alterations in advanced thyroid cancer. In contrast, mutations of PI3K/AKT/mTOR signaling were uncommon in FNAT with prevalence more comparable to TCGA PTC. Similarly, tumor suppressor genes *TP53* and *RB1* mutations were much less common in FNAT than in ATC. This is in agreement with our previous findings on prevalence of PI3K/AKT, *TP53* and *RB1* mutations in ATC (20). Moreover, since *TP53* mutations occur with relative rarity in all histologic types of thyroid cancer except in ATC (20), we anticipate that *TP53* mutations may play a late role in the progression to undifferentiated thyroid cancer. However mutation of *ATM*, another tumor suppressor gene, showed similar prevalence in FNAT and ATC (20). In addition we reported previously a significantly higher mutation burden in *ATM* mutant PDTC and ATC. Presence of mutated *ATM* is therefore consistent with a lack of checkpoint control and DNA repair (27) and its presence in non-anaplastic cancer may predict aggressive behavior before progression to anaplastic carcinoma.

Only 7% of FNAT patients showed chromosomal rearrangements: *STRN/ALK, DLG5/RET* and *OSBPL1A/BRAF* with the latter two representing novel rearrangements not reported before. All rearrangements involved the entire kinase domain of the fusion partner and can therefore lead to a constitutive kinase activation (ALK-, RET- or BRAF- kinase). Chromosomal region where *DLG5* (Disc large homolog 5) gene is located tends to undergo substantial recombination and has a possible role in cell division, proliferation, migration and invasion (28). However this is the first report of *DLG5* as a *RET* partner in chromosomal rearrangements. *DLG5* has a coiled-coil domain which acts as a dimerisation domain for *RET*, similar to other *RET* fusion partners. OSBPL1A *(*Oxysterol-binding protein-related protein 1) is a member of the oxysterol-binding protein family acting as an intracellular lipid receptor. As a result of an alternative transcription start site different OSBPL1A isoforms were found in several cancer types (in colorectal, lung, bladder, liver, prostate, gastric, and brain cancer) (29). In advanced thyroid cancer *OSBPL1A/BRAF* may lead to constitutive activation of BRAF kinase. In *STRN/ALK* coiled-coil domain of *STRN* mediates dimerisation of *ALK* (30). This rare rearrangement has been previously reported in thyroid cancer (18) as well as renal cell carcinoma (31) and colorectal adenocarcinoma (32). *STRN/ALK* fusion represents promissing actionable target since patients with this fusion showed significant initial clinical response to the ALK inhibitors crizotinib and TAE864.

The most common copy number alterations (CNA) in FNAT were gain of chromosome 1q and also loss of chromosome 22. 1q gain was one the most common arm level CNA in our previous report on PDTC whereby PDTC patients with 1q gains had worse survival rates (20). This is in agreement with the high incidence of

1q gain in FNAT. Although more frequent in FNAT patients (26%) than reported for TCGA PTC (15%), 1q gain in PTC is more likely to be associated with more aggressive tumors (33), with significantly higher MACIS scores, higher risk profiles, PTC tumor stage (18) and distant metastases (34). Arm level losses in chromosome 22 were present in 14% of FNAT patients which corresponds with previous reports on TCGA PTC and our PDTC with 22q loss (20). 22q region includes tumor suppressor genes NF2 and CHEK2 (34). NF2 loss promotes *RAS* induced tumorigenesis (22) which is consistent with our previous report on strong association between 22q loss and *RAS* mutated PDTC (20). Since our findings on 1q gain and 22 loss in FNAT correspond with previous reports on aggressive thyroid tumors, these CNA may represent important markers of thyroid cancer aggressiveness.

We identified high prevalence of two mutations in FNAT, which may reflect aggressive behavior: *MED12* (14% of FNAT) and *RBM10* (11% of FNAT). *MED12* (Mediator of RNA polymerase II transcription subunit 12 homolog) is located on X chromosome and encodes for a subunit of the kinase module which is part of the larger macromolecular complex known as Mediator. Mediator complex contains the kinase module and the core Mediator and is important for initiating DNA transcription through interaction with RNA polymerase II (RNA Pol II) (35). Kinase module is necessary for proper Mediator complex function and MED12 plays an instrumental role in the assembly and activation of the kinase module (36,37). Mutated *MED12* can change kinase activity and therefore promote or suppress tumorigenesis depending on the role of the kinase module in a particular tissue (38,39). All *MED12* mutations in FNAT clustered in the same hotspot region of exon 2 resulting in MED12-G44C substitution. This is consistent with clustering of missense *MED12* mutations within hotspots of exon 2 in majority of studies (38), causing alteration of highly conserved amino acid residues (L36, Q43, G44) (40). Therefore, the alteration of a highly conserved residue suggests a gain or change of MED12 function in FNAT. *MED12* showed mutual exclusivity with *BRAF* and *TERT* promoter mutations in our FNAT suggesting their independent oncogenic potential. *MED12* exon 2 mutations were first reported in uterine leiomyomas (UL) (40), followed by breast fibroadenomas, phyllodes tumors and less frequently in uterine leiomyosarcomas, chronic lymphocytic leukemias and colorectal cancers. Due to a significant presence of exon *2 MED12* mutations in specific uterine and breast tumors, further research is necessary to determine its role in the dysregulation of hormonal signaling pathways. Furthermore, expression profiling and gene set enrichment analyses have revealed TGF-β signaling and Wnt/β-catenin signaling in *MED12* mutant UL (38). However further research is necessary to determine the possible role of *MED12* mutations in activating those pathways in thyroid cancer. In addition, *MED12* mutations found outside of exon 2 (in prostate cancer) may act through disruption of CDK8 kinase and subsequent transcriptional dysregulation of p53 and androgen signaling (41,42). Therefore, due to its growing importance, *MED12* has recently been included as a cancer driver gene in a large scale genomic analyses (43,44). In comparison of fatal forms of WDTC and PDTC to nonfatal forms of WDTC and PDTC, mutated *MED12* was more prevalent in fatal forms indicating its importance in tumor aggressiveness. Due to missense *MED12* mutation in FNAT, which causes an alteration of a highly conserved amino acid residue, we anticipate that this mutation may represent a gain or change of function. Nevertheless the complexity of Mediator function and different effect of *MED12* alteration in different tissue types, warrant additional research in order to determine the specific role of *MED12* mutations in FNAT.

RBM10 (RNA Binding Motif Protein 10) may represent another novel tumor marker of aggressiveness, found in 11% of FNAT patients and very rarely in nonfatal counterparts. It encodes for an RNA binding protein which participates in an alternative pre-mRNA splicing. Mutations of genes encoding splicing factors have been implicated in tumor progression (45), since they can directly regulate gene expression and convey phenotypic advantages to select clonal populations. Splicing factors mutations have mostly been found in hematologic malignancies and less frequently in solid tumors (45). *RBM10* mutations have been reported in lung adenocarcinomas (46,47) where they disregulate *NUMB* alternative splicing and cause activation of NOTCH pathway (48,49), critical for lung adenocarcinoma progression. Reports suggest that mutated *RBM10* may act

as an oncogene and wild type *RBM10* as a tumor suppressor gene (46). In addition to missense mutations, truncation mutations with loss of function were also reported in *RBM10* (46). In FNAT, we detected 2 missense and 4 truncating mutations of *RBM10*. We anticipate that *RBM10* mutations in FNAT may represent loss of function in tumor suppression. *RBM10* mutations showed a statistically significant co-occurrence with *NRAS* and *PIK3CA* and a mutual exclusivity with *BRAF* mutations, which suggests mutually independent oncogenic potential of *RBM10* and *BRAF*. Similar to mutated *MED12*, *RBM10* mutations showed a higher mutation prevalence in fatal forms of WDTC and PDTC versus their nonfatal counterparts. In addition, FNAT patients that carried *RBM10* mutations had significantly poorer survival distribution than patients without *RBM10* mutations. Therefore the clustering of mutated *MED12* and *RBM10* within more aggressive thyroid tumors suggests their possible role in predicting tumor aggressiveness.

Conclusions

In this comprehensive clinicopathological and molecular characterization of PDTC we identified distinct features of this uncommon but clinically highly significant type of thyroid cancer. We found that PDTC patients generally display unfavorable clinicopathological characteristics when compared to the majority of well differentiated thyroid cancer patients; older age, male gender, locally advanced initial presentation and tendency for distant spread. pT4a and M1 remained significant predictors of worse outcome in PDTC on multivariate analysis. Undetectable Tg in PDTC patients free of macroscopic disease after initial therapy can predict low risk of recurrence. However due to heterogeneity of PDTC and presence of less differentiated component, these tumors can recur and mandate close follow-up. Initial adequate surgery can achieve satisfactory locoregional control rates, however benefit of currently employed adjuvant treatments is unclear (adjuvant RAI is mostly ineffective in PDTC due to loss of RAI avidity and final conclusions on adjuvant EBRT and chemotherapy are not possible due to small number of patients). Distant metastases represent the major cause of disease specific death in PDTC and since distant disease is not effectively treatable with currently available systemic treatment, development of novel targeted therapies is crucial for improving curability of PDTC. In order to determine the directions for development of targeted therapies, it is necessary to identify drivers of aggressive behavior in PDTC. By using ultra deep next-generation sequencing (NGS), we have identified important biomarkers for aggressive behavior of PDTC (***Table 1***). We found a stepwise increase of mutation burden on thyroid cancer progression scale with intermediate position for PDTC. *TERT* promoter mutations appear with high frequency in PDTC and *TERT* co-occurence with *BRAF* or *RAS* mutations is associated with higher rates of distant spread and a propensity towards greater mortality, possibly associated with positive loop between MAPK stimulated transcription factors and *TERT*. Due to stepwise accumulation of *TERT* promoter mutations and significant presence of *TP53* and PI3K effectors mutations in ATC, these mutations may represent key genetic events distinguishing ATC from PDTC. Among CNA, chromosomal 1q gains represent the most common arm level alterations in PDTC and those patients show worse survival rates. 22q losses are also found in PDTC and have strong association with *RAS* mutation. This is likely due to loss of 22q tumor suppressor gene *NF2* and initiation of *RAS* activation cascade. PDTC also shows mutations in *EIF1AX* translation initiation factor whereby we confirm the presence of unique splice acceptor site specific for thyroid cancer. In addition *EIF1AX* mutations are predictive of worse survival in PDTC. We also analyzed PDTC in the context of fatal cases of non-anaplastic thyroid cancer (FNAT). *TERT* promoter mutation rates and *ATM* mutation rates in FNAT are comparable to ATC. *TERT* promoter mutation is consistent with possible tumor immortalization while *ATM* mutation is consistent with the lack of checkpoint control and DNA repair in aggressive forms of thyroid cancer. We confirm a high incidence of chromosome 1q gain in FNAT as we previously identified in PDTC, which highlights its importance in tumor aggressiveness. We have also identified the presence of 2 novel fusion genes *DLG5/RET* and *OSBPL1A/BRAF* in FNAT. In addition we detected a high

frequency of mutations in novel genes in FNAT not previously reported in differentiated thyroid cancer, *MED12* and *RBM10*, suggesting their role in tumor virulence. When we compared fatal PDTC in our FNAT cohort with nonfatal PDTC identified from PDTC cohort in our previous report (20), fatal PDTC showed a higher frequency of mutations in *TERT* promoter, *MED12*, *RBM10*, *BRAF, HRAS*, TP53, *ATM* and *EIF1AX*, further establishing their role in tumor aggressiveness and progression.

Table 1

Cancer genome alterations in PDTC	% of Tumors
TERT	40
BRAF	33
NRAS	21
EIF1AX	11
PI3K/AKT	11
TP53	8
ATM	7
HMTs	7
RET/PTC	6
SWI/SNF	6
HRAS	5
PTEN	4
ALK	4
PAX8/PPARγ	4
KRAS	2
PIK3CA	2
MED12*	15
RBM10*	12

**novel alterations in fatal PDTC*

We anticipate that this comprehensive characterization of PDTC will aid in the development of risk stratification of PDTC, based on the clinical and genomic parameters, and the development of standard clinical guidelines for management of PDTC. In addition to detection of established genomic markers and commonly disrupted pathways, we have identified novel alterations in PDTC not previously reported in differentiated thyroid cancer. We anticipate that these new insights into PDTC biology will provide incentives and be conducive in narrowing the focus for development of novel targeted therapies with the ultimate goal of improving patient outcomes.

Future Directions

Steady global increase in thyroid cancer incidence over the last few decades has mainly been attributed to the increase in the diagnoses of subclinical well differentiated thyroid cancer (WDTC) (50). Because WDTC represents the most frequent type of thyroid cancer and has a favorable biology, prognosis of thyroid cancer has been excellent with 5 year survival rates of 98% (2). If the rise in incidence of WDTC represents the true rise in incidence (51) and not just overdiagnosis due to greater access to health care (52) or improved technology, we can also expect the rise in the incidence of other more aggressive types of thyroid cancers, like poorly differentiated thyroid cancer (PDTC). Progression model of follicular cell-derived thyroid cancer with progressive dedifferentiation and transition to more aggressive behavior, from WDTC to PDTC and anaplastic thyroid cancer (ATC), is now universally accepted. PDTC is highly significant clinically because it represents the most frequent cause of morbidity and mortality from non-anaplastic thyroid cancer. However there has been little progress in the management strategies of PDTC due to the lack of availability of therapeutic modalities

specific to PDTC. Conventional adjuvant treatment modalities employed for WDTC have failed to improve outcomes in PDTC. Further, there is lack of studies on the biology of PDTC, primarily owing to the relative rarity of the disease. We therefore expect that reports on research studies from tertiary care centers of excellence will contribute to the development of new therapeutic strategies in the management of PDTC, based on risk stratification with clinical and biological parameters. Development of new therapeutic strategies is feasible due to improved understanding of the correlation between clinicopathological features and molecular characteristics of PDTC, particularly in light of rapidly developing new genome sequencing technologies. Diagnostic use of mutational markers as single gene analyses or a panel of mutations has already helped to improve the accuracy of predicting the biological behavior of the cancer on preoperative fine needle aspiration biopsy (FNAB) in differentiated thyroid cancer (DTC) (53). By using the most informative additional gene panels on aggressive clinicopathological behavior it will be possible to preoperatively or postoperatively diagnose and risk-stratify less differentiated tumors, such as PDTC, and escalate the extent of initial treatment and subsequent adjuvant therapies or further treatment during follow-up. In addition, detection of subclonal genomic alterations associated with aggressive behavior, in otherwise WDTC with no other risk factors may become instrumental in predicting and possibly preventing tumor progression.

While locoregional disease can be successfully controlled in the majority of patients with PDTC by adequate surgical treatment, control of distant disease, the leading cause of death in PDTC, has been a problem due to lack of effective systemic treatment. Conventional modes of treatment such as RAI is usually ineffective in PDTC due to loss of differentiation. Adjuvant radiotherapy with or without chemotherapy has been shown to offer significant but short term control of locoregional disease (1). However, this response is unpredictable, not universal and is often associated with significant toxicity. Therefore, the need to develop new targeted therapies is crucial, by employing the translation of molecular genetic data into clinical practice.

Sequencing of the human genome was initially limited to the national level institutions for research purposes only, due to high costs and tedious and slow processing, far out of reach for routine clinical practice. As costs for genomic analysis fell steeply due to new and emerging technologies, sequencing platforms became available at many university core facilities. With the development of high throughput technology, individual research projects have been facilitated and large-scale (consortia-based) projects such as The Cancer Genome Atlas (TCGA) (54) or International Cancer Genome Consortium (ICGC) have been initiated (55). Today, an era of personalized medicine in cancer diagnosis and treatment has become feasible. As opposed to previous sequencing of the most frequently altered regions, or mutational 'hot spots", there is a new rationale in sequencing of tumor samples for the most commonly mutated genes in cancer. They represent "actionable targets" — genes that can either be targeted with drugs or provide clinically relevant information about the disease. One example is Foundation Medicine (56) which offers commercially available genetic profiling of certain types of malignancies at the request of health care providers. Foundation Medicine uses next generation sequencing (NGS) based assays to profile certain solid tumors, such as sarcomas, and hematologic malignancies with more than 300 cancer related genes (56) in order to detect genomic alterations (base substitutions, insertions and deletions, copy number alterations, and rearrangements), as well as select genomic signatures (tumor mutational burden and microsatellite instability). Memorial Sloan Kettering Cancer Center (MSKCC) has launched MSK-IMPACT assay on NGS platform (57), which is even more inclusive and can be used on any solid tumor type regardless of its origin. MSK-IMPACT has been validated for high accuracy and sensitivity and currently examines 410 cancer related genes with regular revisions of the gene panels. This test is currently available only for MSKCC patients, however, MSK-IMPACT will help further the development of "basket studies". Basket studies are clinical trials of targeted therapies in patients whose tumors test positive for particular mutations, regardless of the tumor type or origin. By greater number of patients included, basket studies can provide more information than the traditional disease- specific trials and

may significantly accelerate progress in precision therapy development. Through results of MSK-IMPACT assay, patients can be quickly matched with available targeted therapy or a specific basket study. Basket studies are therefore designed as molecular allocation studies (57) in order to test anticancer therapies. This facilitation of patient inclusion into the basket study is particularly useful for rare tumors or tumors with rare mutations (57). Examples are low prevalence mutations in thyroid cancer, such as rearrangements of e.g. *RET*, *NTRK1*, *NTRK3* or *ALK*. These types of thyroid cancers could be included in trials with selective kinase inhibitors that have shown efficacy in other types of cancer. In addition, the data and analyses of MSK-IMPACT and basket studies are shared with the scientific community and greater public (58). Particularly good candidates for novel targeted therapies in thyroid cancer are patients with very low survival rates, characterized by distant metastasis with RAI resistant thyroid tumors and positive 18FDG-PET scan (59), unresponsive to traditional modes of adjuvant treatment. Novel targeted therapies will also benefit patients with progressive disease (defined by RECIST criteria (60)), symptomatic disease, those with large tumor burden and patients with tumor at surgically less accessible locations and/or locations in proximity of vital structures (e.g. trachea, spinal cord, brain) (59).

The majority of studies on thyroid cancer targeted therapies have been focused on small molecules such as tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs), with promising results. Both types of molecules inhibit tyrosine kinase activity by binding to one or multiple tyrosine receptor kinase (TRK) (61). When compared to TKIs, mAbs are unable to penetrate cell membrane (59) and will probably be more adequate for use against circulating cancer cells than against solid tumors. The research on thyroid cancer treatment has been therefore more focused on TKIs. In addition, TKIs have been the most studied drugs in thyroid cancer because of their direct effect on the most commonly activated signaling pathways. The primary target of TKIs is catalytic activity of TRK, more specifically competitive ATP inhibition at the catalytic binding site of tyrosine kinase. This results in a cessation of the proliferative signal. TKIs are generally multitargeted, acting on multiple participants of the signaling cascade (except selumetinib which binds only MAPK kinase (MEK), decreases extracellular signal-regulated kinase (ERK) and restores ^{131}I uptake) (19,59,62). Multiple clinical trials on TKIs in thyroid cancer have been concluded and several are still ongoing and/or actively recruiting patients (59). In the United States, Food and Drug Administration (FDA) has so far approved several TKIs for treatment of medullary thyroid cancer. However, only sorafenib and lenvatinib have been approved for thyroid cancer of follicular cell origin. These two drugs have been approved for progressive, recurrent, or metastatic disease that does not respond to radioactive iodine (63) based on phase III, prospective, randomized, double blind and placebo-controlled trials (64,65). Of particular interest has also been the restoration of RAI uptake through inhibition of MAPK signaling. Selumetinib (MEK inhibitor) restored RAI uptake successfully in a subset of patients with metastatic RAI refractory thyroid cancer (66). Dabrafenib (BRAF inhibitor) has achieved similar effects in another study (67). Ongoing randomized phase II trial is examining the effect of RAI with or without selumetinib in treatment of patients with recurrent or metastatic thyroid cancer (ClinicalTrials.gov identifier: NCT02393690) (68). Interestingly phase III studies do not strongly support the hypothesis that patients whose tumors contain mutations of TKIs main targets (e.g. BRAFV600E for sorafenib) show better response to treatment with TKIs than those without those mutations (59). Progression free survival by TKIs has been demonstrated in many phase III studies, however survival improvement by TKIs could not be extrapolated based on the present studies (59) and it should be determined in future studies on TKIs. Despite promising results of clinical trials involving TKIs, there are several obstacles to the efficiency of TKIs therapy that need to be addressed in the future. TKIs are generally cytostatic and not cytotoxic whereby tumor cells are not destroyed but rather put into quiescent state. This is why complete response is not achievable, but only partial response can be obtained which is mediated through antiangiogenic actions and tumor ischemic necrosis (59). Additional disadvantage of TKIs is that they should be administrated until tumor progression or resistance occurs, because once TKIs are stopped, tumor progression can become even more rapid (59) likely due to unleashing of resistant clones. In addition, toxicity of current TKIs can significantly impede therapy

maintenance and thus therapy efficiency. In order to improve the quality of life in patients treated with TKIs, clinical studies will need to determine the optimal time to start the treatment (e.g. at very early or advanced stages) (59). Negative cumulative effects of long term therapy with TKIs need also to be examined in future studies. Furthermore, unselective effects of TKIs caused by " On-target" toxicity (blocking of common target for neoplastic and normal cells) or " Off-target" toxicity (inhibition of non intended target) (69) need to be addressed, possibly with development of "advance precision" TKIs. However, the main challenge in targeted cancer therapy is to overcome resistance in order to achieve long term successful outcomes. Resistance to TKIs (e.g. sorafenib, vandetanib, or lenvatinib) has been reported in thyroid cancer (59). Besides primary resistance (no initial response to treatment), secondary resistance can occurr at variable time from initial response to therapy ("escape phenomenon"). Research shows that the most plausible explanation of the latter is the development of specific mechanism by which tumor cells "escape" and become unaccessible to particular treatment. Tumor can bypass the action of the drug via secondary sites of mutations, probably located downstream from TKI target or in the parallel pathways (59). In order to prevent the development of resistance, the paradigm of treatment is shifting towards targeting of multiple pathways simultaneously. This would disable the activation of an alternative pathway after blocking of a single pathway. In addition to resistance prevention, dual therapy strategy may result in a synergistic effect of targeting the multiple pathways simultaneously. This is particularly important in development of therapies for more aggressive thyroid cancers such are PDTCs and ATCs, since multiple pathways are found to be simultaneously activated in these advanced cancers (70). Indeed dual inhibition of the MAPK and mTOR pathways or the MEK and mTOR pathways resulted in a strong inhibitory synergism in thyroid cancer cell lines, including those from ATC (71,72), similar to the effect of dual inhibition with RAF and PI3K/mTOR in DTC cell lines (73). In addition, simultaneous inhibition of multiple other targets are being considered, such as dual inhibition of histone deacetylases (HDACs) and PI3K/AKT pathway (74), BRAF and MEK inhibition (75) or BRAF and EGFR inhibition (76). In terms of functional restoration, simultaneous inhibition of MAPK, PI3K/AKT and histone deacetylase pathways could increase iodide uptake and radioiodine avidity due to re-expression of genes responsible for iodide incorporation, which could be additionally enhanced with co-treatment with TSH (77). Another treatment strategy that could overcome drug resistance and/or improve drug efficacy is to combine targeted drugs with traditional modes of therapy such as chemotherapy or radiotherapy. Combination of dual inhibitor of PI3K/mTOR (BEZ235) with paclitaxel, imatinib and docetaxel, and combination of PPARγ agonist efatutazone with paclitaxel showed synergistic effects in vitro compared to the effect of single agent treatment (78-80).

In addition to RTKs, other oncogenic drivers of thyroid cancer progression are being considered for targeted therapy. Proteasome inhibitor bortezomib prevents inhibitory-kappa B degradation and thereby blocks constitutive activation of NF-κB pathway and cell proliferation. Bortezomib also increases expression of TNF-related apoptosis-inducing ligand (TRAIL) which activates caspases and can be used to induce apoptosis in thyroid cancer (70,81). Cancer adaptation to hypoxia and angiogenesis has also become an important target of thyroid cancer therapy research. In response to hypoxia, HIF1α transcription factor induces genes responsible for cell metabolism and angiogenesis via VEGF overexpression (82). In addition to hypoxia, growth factor signaling pathways PI3K/AKT and MAPK can also upregulate HIF1α (70). The majority of TKIs (including FDA approved sorafenib and lenvatinib) block receptors of VEGF (VEGFRs), however cabozantinib may be the most promising in targeting angiogenesis since it blocks both VEGFR and HIF1α signaling (59). Since HIF1α is only expressed in thyroid cancer cells and not in normal thyroid tissue (83), this marker can represent a paradigm of precision medicine with further research focused on drivers specifically expressed in cancer tissues. Tumor angiogenesis can also be targeted by microtubule-depolymerizing agent, such as combretastatin A-4 phosphate (CA4P) (84), which induces ischemic necrosis by impairing tumor vasculature function (59). Clinical trials currently also focus on multitargeted kinase inhibitors, which simultaneously inhibit angiogenic targets and targets in MAPK pathway (85). In addition, antiangiogenic agents could increase the efficacy of

conventional therapies (chemotherapy, radiotherapy or RAI treatment) (86). Clinical trials on modulators of growth, apoptosis, or other novel targets are currently pending.

The use of mAbs in the ever growing field of potential targets in thyroid cancer is yet to be explored. So far, mAbs have been studied in relation to tyrosine kinase receptors or their ligands, mostly VEGF, in order to achieve antivascular effect (87). In addition, mAb may be instrumental in delivering different modes of treatment and thus improving their effectiveness or precision; e.g. for precise delivery of radioactive therapy use of conjugated mAb with radioisotope that is directed against carcinoembryonic antigen (CEA) on metastatic thyroid cancer cells (88).

Insights into the comprehensive genomic profile of PDTC offer new targets for development of precision therapies. *TERT* promoter mutation represents an attractive target due to its association with immortal phenotype and development of distant metastases, responsible for disease specific deaths in PDTC. There has been a renewed interest in anticancer therapy of *TERT* promoter mutated tumors. One rationale is that mutated TERT represents a near-ubiquitous tumor antigen that is expressed at practically every stage of tumor evolution (89). Furthermore, mutation of *TERT* promoter leads to transcriptional upregulation of *TERT* and a high TERT-protein expression, which could increase TERT-antigen presentation by cancer cells with increased susceptibility to T-cell recognition and attack (89). This is a likely scenario because there is ample evidence of the functioning antigen-processing machinery at various stages of tumor evolution (89). The effectiveness of TERT vaccination might be augmented by simultaneous immune-checkpoint inhibition, with enhanced T-cell responses. In addition, TERT vaccination might increase the response rates to immune-checkpoint inhibitors thus expanding the indications for the type of cancers that can be treated by immune-checkpoint inhibitors (89). Another actionable alteration is *STRN-ALK* rearrangement, which could be targeted with ALK inhibitors, crizotinib and TAE864 (30). Furthermore, mutations in the Mediator kinase module, especially those of *MED12*, represent a promising target due to the significant role of *MED12* in cell transcription. So far attempts to target the kinase module have been mostly directed towards CDK8 kinase with e.g. sorafenib as a multi tyrosine kinase inhibitor/CDK8 inhibitor and Senexin A as a novel CDK8/19 inhibitor (38). Future studies will need to look closely at the effects of targeting the Mediator kinase module in different tissues due to its variable effect on both repression and activation of transcription (38,90). Novel therapeutic strategies at the level of transcription may also target mutated *RBM10*. Promising results have been reported from a phase I trial of spliceosome inhibitor E7107 in patients with advanced solid tumors unresponsive to standard therapies (91). Splicing factor inhibitors may thus prove useful in patients with *RBM10* mutations.

The current goal of precision therapy is to genomically profile individual patient's tumor, in order to apply targeted treatment with most effectiveness and minimal adverse effects. Future studies will need to further investigate the benefits and the mode of combining different targeted therapies or targeted therapies combined with traditional ones. It is important to clarify whether to apply different therapies simultaneously to maximize their effect, to lower the doses of simultaneously applied therapies in order to decrease toxicities, or is it more beneficial to apply different types of treatments in a successive manner. Future studies will also need to determine cross-resistance between drugs in order to avoid unnecessary and potentially harmful treatments (59). In addition, the introduction of cost-effective genomic sequencing into a routine clinical practice will possibly evolve into a dynamic genomic sequencing. Genomic analysis of a patient's tumor during the course of treatment and follow-up will become the standard of care. In case of resistance to the drug, responsible mutations will be determined and new drugs will be introduced or new clinical trials initiated with the aid of genomic information. These new developments will be made possible through continuous evolution of new sequencing technologies. In recent years, introduction of single-cell sequencing has been an important breakthrough in sequencing technologies. It was first reported in 2009 as single-cell RNA sequencing (92)

followed by single-cell DNA sequencing in 2011 (93). Single-cell sequencing includes isolation of individual viable cells or intact nuclei, amplification of DNA or RNA from the cell and sequencing on appropriate NGS platform (94). Up to that point most technologies have required DNA or RNA from over 100 000 cells (95) and sequencing has been performed on a bulk of cells with insight into overall molecular characteristics of the tumor. However, solid tumors are often admixtured with more than 50% of non-cancerous cells such are fibroblasts, endothelial cells, lymphocytes and macrophages, which can significantly mask the detection of signal belonging to the cancer cells (95). In addition, solid tumors often include multi-clonal subpopulations which can influence the pathways of cancer progression (96). Since no single cells are being profiled, traditional "bulk sequencing methods" cannot provide true information on tumor genetic heterogeneity. Single-cell sequencing may therefore further improve our understanding on tumor biology and progression. It may also facilitate inter- and intra-tumor comparisons and improve molecular classification methods (95). In the near future single-cell sequencing may significantly improve diagnosis, especially early detection of rare tumor cells (e.g. in fine-needle aspirates) and monitoring of circulating tumor cells (95). Furthermore, single-cell sequencing may also become instrumental in selection and evaluation of cancer treatment with targeting of the "driver" as well as resistant clones. Established platforms of second generation sequencing offer cost-effective output for single-cell sequencing. Future technological development might facilitate convergence of single-cell sequencing with third generation single molecule technologies. Third generation sequencing has the potential to maximally utilize the high catalytic rates and processivity of DNA polymerase without the use of chemical reagents (97) in order to radically increase throughtput, read length, speed, consensus accuracy especially for rare variant detection, with further lowering of costs (98). Such progress of sequencing technology will further improve our understanding of genome, epigenome and transcriptome. Development of sensitive single molecule methods that perform sequencing without the need for pre-amplification will facilitate more comprehensive analysis of DNA modifications (e.g. methylation or irradiation or chemically induced), because template DNA modifications affect DNA polymerase activity during the amplification (99).

Furthermore development of long-read sequencing will contribute to RNA structure analysis and facilitate analysis of long complex segments such as segmental duplications or CNA (99). Improved characterization of small non-coding miRNA implicated in the regulation of protein translation is to be expected, especially with acquisition of whole genome miRNA profiles (97). Indeed with further steep decrease in sequencing costs, it will become cost-effective to perform the whole genome sequencing rather than sequencing of targeted exonic regions (100). This is significant since the majority of the human genome consists of non-coding regions, including gene promoters, enhancers, and intronic regions (101), such as *TERT* promoter mutations that has been associated with progression of thyroid cancer (102). In the future all these advances may significantly complement the signature biomarker profile of thyroid cancer progression.

However, the fast development of sequencing technologies, capable to create Mb or Gb of data per sequencing reaction, needs to be accompanied by cost-efficient storing and processing of huge amounts of data. Currently data processing and storage represent the main limit of further expansion of novel sequencing technologies. NGS platforms require support of information technology through all stages of sequencing, data analysis, display and integration, including quality control, storage, and data tracking (103). Therefore, in order to avoid future bottleneck formation, progress in information technology with new analytic approaches in mining the NGS datasets (104) must follow the pace of data supply. Indeed, the new era of personalized precision medicine has been launched. We are witnessing the evolution of deep molecular scanning which will become routine in the future. Through further correlation of clinicopathologic expression of the disease with underlying genomic events we will continue to advance our insights into the biology of the disease. This will eventually result in shifting the traditional modes of treatment from treating the symptoms towards treating the origin and drivers of the disease.

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Chapter 11

Summary

Summary

Poorly differentiated thyroid cancer (PDTC) occupies clinically and histopathologically an intermediate position on a progression spectrum from differentiated thyroid cancer (DTC) to anaplastic thyroid cancer (ATC). However, studies on PDTC have been limited due to relative rarity of the disease and heterogeneous diagnostic criteria. In this work we present a comprehensive study of one of the largest cohort of PDTC patients reported in the literature, diagnosed by criteria of proliferative grading and treated surgically at a tertiary care academic center (Memorial Sloan Kettering Cancer Center; MSKCC), with or without adjuvant therapy during the period of over 24 years. The main objectives of our study were to report on clinicopathological, biochemical and genomic characteristics of PDTC patients, to identify the patterns of treatment failure and to correlate clinicopathological, biochemical and genomic characteristics with outcomes.

PDTC patients tended to present with clinicopathological characteristics that represent adverse prognostic factors in thyroid cancer: older age, higher prevalence of male gender, gross extrathyroidal disease, regional neck metastasis and distant metastasis. Initial surgery resulted in satisfactory 5 year locoregional control rates (81% in PDTC and 56% in PDTC with gross extrathyroid extension (ETE)). Majority of PDTC patients received adjuvant therapy of radioiodine (RAI) and/or external beam radiation therapy (EBRT), however final conclusions on the benefit of adjuvant therapy on disease control are not possible due to small number of patients. We also examined postoperative thyroglobulin (Tg) levels in PDTC patients and found that PDTC patients with undetectable Tg have low risk of recurrence. This finding has to be interpreted in the light of tumor heterogeneity in PDTC and presence of less differentiated component, which warrants close follow-up of PDTC patients. 5 year disease specific survival of PDTC was low (66% in total PDTC and 49% in PDTC with ETE). Indeed, our study showed that PDTC is a highly significant entity, responsible for majority of deaths (57%) from fatal non-anaplastic thyroid cancer. We found that PDTC patients that died of the disease compared to those that survived were: significantly older, presented with larger tumors, local invasion, higher stage and distant metastases. pT4a and M1 stood as significant predictors of worse outcome on multivariate analysis. We identified distant disease as the major cause of death in PDTC patients. Therefore detection of molecular drivers of the disease and development of novel systemic targeted therapies are neccessary in order to control disease progression and improve overall outcomes.

In order to identify molecular drivers of the disease, we studied genomic profile of PDTC patients. For the first time we report a high frequency of *TERT* promoter mutations in follicular cell-derived thyroid cancer. In addition, overrepresentation of *TERT* promoter mutations in PDTC when compared to PTC may signify a novel biomarker of thyroid cancer progression. *TERT* promoter mutations were significantly associated with *BRAF* or *RAS* mutations in PDTC and ATC, consistent with de novo consensus binding sites in *TERT* promoter for MAPK activated ETS factors. We used ultra deep next-generation sequencing (NGS) technology and MSK-IMPACT targeted assay to profile 341 cancer relevant genes in PDTC. When sequencing of PDTC was compared to the results from sequencing of anaplastic thyroid carcinoma (ATC) and papillary thyroid carcinoma (PTC) of TCGA study, we found genomic instability and accumulation of mutations with thyroid cancer dedifferentiation. This was also accompanied by accumulation of aggressive clinical characteristics and reduced overall survival. Contrary to *BRAF* mutations, *RAS* mutations were more prevalent in advanced tumors in comparison to PTC. *BRAF* mutated PDTC showed significantly higher rates of regional nodal metastases while *RAS* mutated PDTC showed significantly higher rates of distant metastases, consistent with reports on DTC. BRAF-RAS score (BRS), derived from expression patterns of gene panels for BRAFV600E and *RAS* mutated tumors, was preserved in PDTC, i.e. correlated with *BRAF or RAS* mutational status, as shown for PTC. Similarly, we found preservation of thyroid differentiation score (TDS) expression panel in relation to the mutated drivers in PDTC as in PTC. Furthermore, we found the stepwise increase in *TERT* promoter, *TP53* and PI3K pathway effectors mutations

between PDTC and ATC. These mutations may therefore represent key genetic events in progression between PDTC and ATC. Copy number alterations (CNA) analysis revealed 1q gain as the most common arm level CNA in PDTC whereby PDTC patients with 1q gains had worse survival rates. In case of PDTC with 22q loss, strong association with *RAS* mutation was present. This can be explained by the transcriptional activation of RAS after the loss of 22q tumor suppressor gene. We also detected mutations of translation initiation factor gene *EIF1AX* in PDTC with comparable frequencies to ATC. *EIF1AX* mutations predicted for worse survival in PDTC patients and may represent a useful marker for risk stratification of PDTC. We also performed ultra deep NGS of PDTC in the context of fatal non-anaplastic thyroid cancer (FNAT). The most common mutations in FNAT were those of *TERT* promoter. The co-occurrence of *TERT* mutations with *BRAF* or *RAS* mutations in FNAT was consistent with our previous findings in PDTC and ATC. Alterations of MAPK pathway (*BRAF* and *RAS*), *EIF1AX* and *ATM* showed similar prevalence in FNAT compared to ATC, indicating their importance in aggressive thyroid cancer. High incidence of chromosome 1q gain was present in FNAT, consistent with findings of worse survival rates in PDTC with 1q gain. Sequencing of FNAT also revealed presence of novel alterations: fusions *DLG5/RET* and *OSBPL1A/BRAF* and high frequency of *MED12* (14%) and *RBM10* (11%) mutations, suggesting their role in tumor fatality. Comparison between fatal PDTC and nonfatal cases of PDTC revealed that fatal PDTC show a higher frequency of following mutations: *TERT* promoter, *MED12*, *RBM10*, *BRAF, HRAS*, *TP53*, *ATM* and *EIF1AX*, highlighting their role in thyroid cancer aggressiveness and progression.

In the present study we report on a comprehensive characterization of PDTC patients, with emphasis on clinicopathological and molecular markers predictive of poor outcome. In addition we report distant disease as the main cause of disease related mortality in PDTC patients. We anticipate that these insights into PDTC biology will contribute to the development of standardized clinical guidelines and development of effective systemic targeted therapies in order to improve the outcomes in patients with PDTC.

Chapter 12

Appendices

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CURRICULUM VITAE

Tihana Ibrahimpašić was born in Zagreb, Croatia and holds a Doctor of Medicine degree from the University of Zagreb School of Medicine, Croatia and from Karl Franzes University of Graz, Austria. Following a one year internship in general medicine and general surgery, she completed the State Licensing Exam of Croatia. She was then accepted into a residency program in otorhinolaryngology at the University Hospital Center Zagreb in 2004, from which she graduated with the Board exam in 2009.

During the residency training, she participated in a scientific project on early detection of laryngeal cancer and enrolled in a two-year postgraduate studies in Biomedicine at the University of Zagreb, which included studies in basic medical sciences, translational research and public health. In addition, during the residency training, she also worked on the teaching staff at the University of Zagreb School of Medicine, holding lectures and practical seminars in otorhinolaryngology for medical students.

Among fellowships and acknowledgments she received for academic excellence are fellowships from the Austrian Ministry of Science and from the Union for International Cancer Control (UICC). In 2007, through UICC ICRETT (International Cancer Research Technology Transfer) fellowship, she participated in a three-month clinical observership and in a project on multidisciplinary treatment of advanced head and neck cancers as a visiting resident at Memorial Sloan Kettering Cancer Center (MSKCC), New York. Upon return to the University Hospital Center Zagreb, she completed the residency and worked as a specialist in otorhinolaryngology. In 2011 she returned to MSKCC, New York to work as a clinical research fellow on a scientific project on poorly differentiated thyroid cancer (PDTC) in order to pursue a PhD degree. Her initial work included development of an institutional PDTC database, which formed the basis for the research on clinico-pathological characteristics of PDTC presented in this thesis. The second part of her project included genomic profiling of PDTC within MSKCC Human Oncology and Pathogenesis Program, where she participated in laboratory work and data analysis of PDTC genomic sequencing by Sequenom MassARRAY iPLEX platform and ultimately by an ultra deep next-generation sequencing platform MSK-IMPACT.

With the mentorship of the senior faculty, she produced a series of peer reviewed studies on clinical and molecular characteristics of PDTC, which ultimately resulted in the production of this thesis. These studies have been presented at major international conferences and she is the recipient of the best research poster award from the American Head and Neck Society, at the 8th American Head and Neck Society International Conference.

She is a member of the American Thyroid Association and an invited member of UICC reviewer panel for ICRETT fellowships. She speaks English, German and Croatian fluently. In her free time she plays piano and studies Brazilian martial art Capoeira.

PORTFOLIO

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Dr. I. Ganly, Dr. R. A. Ghossein

Courses

2011 **Biostatistics Course with final exam,** Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, NY, USA

2011 **How to Develop A Successful Research Proposal (Strengthening Your Proposals/Proposal Development: From Idea to Award/ Foundations)**, Tri-Institutional Collaboration Network (TCN) NY, USA

2011 **Investigator Training Program: Facilitating Global Excellence in Clinical Trials**, Weill Cornell Medical Center, Clinical & Translational Medical Center NY, USA

2011 **Ethical conduct in research, Collaborative Institutional Training Initiative and HIPAA Compliance certificate (**Health Insurance Portability and Accountability Act-Protection of Patient Privacy: Medical Records and Personal Health Information**),** Memorial Sloan Kettering Cancer Center, NY, USA

2012 **Writing for Biomedical Publication,** Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, The New York Academy of Sciences, Tri-Institutional Collaboration Network (TCN), NY, USA

2012 **Essential Strategy for Writing Successful Grants (Finding the Right Funding/An Inside Look at Grant Reviews/Structure of Your Proposal/Writing Your Specific Aims)** Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, NY, USA

Seminars, workshops and masterclasses

2011 **Science in the Clinic (The Research Experience/ Writing & Submitting a Paper for a Peer Reviewed Life Sciences Journal)** ATA Fellow grant participant, 81st Annual Meeting of the American Thyroid Association, Indian Wells, USA

2011 **SPARC Conference 2011**: **Achieving Successful and Productive Academic Research Careers**, Rockefeller University, NY, USA

2012 **Presenting to an Audience Outside Your Field** ATA Fellow grant participant, 82nd Annual Meeting of the American Thyroid Association, Quebec, Canada

2012 **Scientific Paper Writing and Presenting Workshop,** Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center**,** NY

2012 **Grant writing seminar and workshop,** Weill Cornell Medical Center, Clinical & Translational Medical Center NY, USA

2012 **Academic Publishing in Sciences** by Elsevier executive/ **How to Get Published** & **Publishing Ethics,** Sloan-Kettering Institute, Office of Postdoctoral Affairs, NY, USA

2012 **Memorial Sloan Kettering Cancer Center Central Library /The Corporate Division of TCCIT (The Career Center Informational Technology) training workshops:** PubMed Biomedical Sciences, EndNote, Poster Creation

2012 **Department of Surgery Summer Weekly Clinical Research Methods Series**

- Immunotherapy trials in cancer
- Qualitative methods in clinical research
- Disparities and Diversitiy in Research
- Medical informatics
- Genomic profiling
- Clinical Trials
- ROC curves
- Behavioral Research Methods and Patient Reported Outcomes

2013 **Department of Surgery Summer Weekly Clinical Research Methods Series**

- Correlative studies involving genomics
- Phase I-III clinical trials
- Population based data analysis
- Quality of life in clinical trials
- Research ethics
- Surgery and Databases
- Survival and Elements of Study Design
- Univariate and Multivariable Analysis

Professional Courses & Conferences in Head and Neck Diseases

2011 **Current concepts in Head and Neck oncology and surgery**, Memorial Sloan Kettering/University of Toronto/MD Anderson, NY, USA

2011 **ATA Fellow grant participant & Ultrasound course**, 81st Annual Meeting of the American Thyroid Association, Indian Wells, USA

2011 **Laryngeal cadaver course,** San Francisco, USA

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2012 **Diagnostic Endocrine Neck Ultrasound and UGFNA Course**, AAEC-American Association of Clinical Endocrinologists, Philadelphia, USA

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2013 **Current concepts in Head and Neck oncology and surgery**, Memorial Sloan Kettering/University of Toronto/MD Anderson, NY, USA

Head and Neck Weekly Multidisciplinary Conference, Memorial Sloan Kettering Cancer Center, NY, USA

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Presentations

Head and Neck Weekly Multidisciplinary Conference, Memorial Sloan Kettering Cancer Center, NY, USA; oral podium presentations:

2011 Poorly differentiated thyroid carcinoma presenting with gross extrathyroidal extension: 1986-2009 Memorial Sloan Kettering Cancer Center experience

2012 Undetectable thyroglobulin after total thyroidectomy in patients with low- and intermediate-risk papillary thyroid cancer; is there a need for radioactive iodine therapy?

2012 Prognostic implications of papillary thyroid carcinoma with tall-cell features.

Congress visits with presentations:

2011 **81st Annual Meeting of the American Thyroid Association**, Indian Wells, CA, USA

Poorly differentiated thyroid carcinoma presenting with gross extrathyroidal extension: 1986-2009 Memorial Sloan Kettering Cancer Center experience (oral podium presentation)

2011 **53rd Annual Meeting, ASTRO- American Society for Radiation Oncology**, Miami Beach, Florida, USA

Role of Postoperative Radiotherapy in Management of Well Differentiated Thyroid Carcinoma with Gross Extrathyroidal Extension: Memorial Sloan Kettering Cancer Center Experience (poster presentation)

2012 **American Association of Endocrine Surgeons Annual Meeting**, Iowa city, Iowa, USA

Undetectable thyroglobulin after total thyroidectomy in patients with low- and intermediate-risk papillary thyroid cancer; is there a need for radioactive iodine therapy? (oral podium presentation)

2012 **8th International Conference on Head and Neck Cancer, AHNS**, Toronto, Canada

Prognostic implications of papillary thyroid carcinoma with tall-cell features (poster presentation)

**American Head and Neck Society (AHNS) award for the best poster presentation at the 8th International Conference on Head and Neck Cancer, AHNS, 2012, Toronto, Canada*

2012 **82nd Annual Meeting of the American Thyroid Association**, Quebec, Canada

Outcomes in patients with poorly differentiated thyroid carcinoma (poster presentation)

2013 **2nd World Congress on Thyroid Cancer**, Toronto, Canada

Mutational landscape of poorly differentiated thyroid carcinomas (oral podium presentation)

2013 **83rd Annual Meeting of the American Thyroid Association**, San Juan, Puerto Rico

Mutational landscape of non-anaplastic thyroid carcinomas that died of the disease (poster presentation)

2013 **New York Thyroid club, Lenox Hill hospital, NY, USA**

Mutational landscape of thyroid carcinomas (oral podium presentation)

2014 **5th World Congress of IFHNOS & Annual Meeting of the AHNS**, NY, USA

Postoperative thyroglobulin levels in patients with poorly differentiated thyroid cancer -are they useful? (oral podium presentation)

List of publications

Outcomes in Patients with Poorly Differentiated Thyroid Carcinoma
***Ibrahimpasic T**, Ghossein R, Carlson DL, Nixon I, Palmer FL, Shaha AR, Patel SG, Tuttle RM, Shah JP, Ganly I.*J Clin Endocrinol Metab. 2014;99(4):1245-52

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ACKNOWLEDGMENTS

There is no such thing as a one woman endeavor. I would like to express my gratitude to all who contributed to this work and made this thesis possible. This has been a period of intense learning and development of new perspectives and I would like to reflect on the people who encouraged me and helped me on this path.

First and foremost I am indebted to **Prof. dr. Jatin Shah** for his invaluable and continuous guidance. It is a once in a lifetime opportunity and privilege to work with and learn from a person of such excellence, who represents an exemplary role model both on a professional and human level. His energy, vision, immense knowledge that he is always willing to share and genuine interest in other people's development are truly inspiring. He showed me what true leadership looks like, where substantial impact on others is inevitable. He taught me the skills, that fundamentally changed my views, on how to: challenge my thinking, ask critical questions, solve problems and view issues from multiple perspectives. I am truly grateful for his guidance that was crucial in my training and development.

I am particularly indebted to **Dr. Ian Ganly** for his guidance throughout the course of our studies. His knowledge, creativity and outstanding productivity, as well as willingness to teach others, are truly inspirational. I have been presented with numerous opportunities for learning and growth because of his ability to efficiently tackle diverse intellectual problems. He has taught me invaluable lessons in how to approach a scientific question, design the study, apply the methodology to carry out the research and how to present the research works in a clear and concise manner. I will always be grateful for his mentorship and for the valuable knowledge and concepts he taught me.

My special gratitude goes to **Dr. Ronald Ghossein** for his guidance and mentorship on the clinicopathological aspects of our research. His enthusiasm and energy are contagious and his expertise and thoroughness of pathological classifications were essential for the qualities of our studies. His generous sharing of time, ideas and knowledge throughout our research was invaluable and I am profoundly grateful for that.

My heartfelt thanks go to **Prof. dr. Alfons Balm** whose guidance, enthusiasm and encouragement throughout the research studies and thesis preparation were nothing short of amazing. I am profoundly grateful for his advice and directions that were indispensable in project completion and thesis preparation.

I am particularly grateful to **Prof. dr. James Fagin** for the opportunity to conduct the research in his world-class thyroid lab, where I learned all the necessary techniques, important genomic concepts and acquired new understandings through his weekly scientific lab meetings. Through his generous mentorship a new world of molecular analysis has been opened to me and I am very grateful for that.

I would like to express my gratitude to the experts who co-mentored me on the clinical studies. They are not only highly knowledgeable and insightful but also wonderful and amazing people who make learning and solving problems fun. I am indebted to **Prof. dr. Snehal Patel**, **Prof. dr. Ashok Shaha** and **Prof. dr. Michael Tuttle** for their constructive insight and advice, which were vital for development of my critical thinking and understanding of scientific questions from multifaceted perspectives.

I am very grateful to **Dr. Diane Carlson** for generous sharing of her time and expertise in the histopathological review and classification of our samples. Her contribution was essential for the quality of our research and I will always feel deep admiration for her work and mentorship.

My deep appreciation goes to **Frank Palmer** whose knowledge and excellent understanding of research methodology and biostatistics were precious and indispensable in the development of our studies and in

discussions during data analysis. My sincere thanks go to **Monica Whitcher** for her important contribution in data analysis and publication.

To **Jose Dominguez** and **Julio Ricarte-Filho** I owe special gratitude for their generosity in mentoring me through the lab work and for their energy and openness for discussion of any questions. I admire their curiosity and enthusiasm to clarify and examine various ideas and concepts. The learning curve of important research aspects would be much steeper for me without fruitful and engaging discussions we had. I am also grateful to **Jeffrey Knauf** and **Cristina Montero-Conde** for their kindness and readiness to discuss any questions about technical aspects of lab research or about general concepts in molecular biology research.

My sincere thanks go to my co-authors; without them the depth and quality of our research would not be the same. I am grateful to **Bin Xu** and **Iñigo Landa** for their energy, knowledge and persistence invested in pursuing the common goals. I am indebted for the insightful genomic analysis and indispensable advice generously given by **Timothy Chan**, **Michael Berger**, **Luc Morris**, **Barry Taylor**, **Chris Sander**, **Rileen Sinha**, **Venkatraman Seshan** and **Nikolaus Schultz**. I am profoundly grateful for all the people involved in our studies, who invested their time, intellect and creativity at the highest level while teaching me a lot along the way: **Iain Nixon**, **Nancy Lee**, **Laura Boucai**, **Snjezana Dogan**, **Laura Wang**, **Mona Sabra**, **Brian Untch**, **Jocelyn Migliacci, Natalya Chernichenko, Norisato Mitsutake, Michiko Matsuse**, **Ronak Shah**, **Gnana Krishnamoorthy**, **Sumit Middha** and **Shyam Deraje**.

My time at Memorial Sloan Kettering Cancer Center was a blessing of its own and I am thankful for the atmosphere of learning and accomplishment. I am grateful for all engaging lectures, courses, seminars and workshops I attended, where I learned from the extraordinary experts that generously shared their knowledge. My sincere thanks go to the excellent and knowledgeable library staff, for their educational modules on the databases and data search and their kindness and readiness to assist at any time.

I would like to express my sincere appreciation towards the members of the Doctorate Committee, for their time and effort in reviewing the thesis manuscript: **Prof. dr. M.W.M. van den Brekel**, **Prof. dr. E. Fliers**, **Prof.dr. R.J. Bennink**, **Prof.dr. L.E. Smeele**, **Prof.dr. M.J. van der Vijver**, **Dr. E.J.M. Nieveen van Dijkum** and **Dr. V.B. Wreesmann**.

I am forever indebted to my family for their unparalleled love and support and to them I dedicate this work. I am thankful to my brother **Haris** for his invaluable support, encouragement and inspiration based on his own professional accomplishments. I am grateful to my parents who raised us with the love for knowledge and supported our natural curiosity. The achievements of my Mom, an accomplished biotechnology expert and Dad, a recipient of Nikola Tesla award for patents in applied mathematics and physics, represent the ultimate role models for me. Thank you **Mom** and **Dad** for the love, experiences and opportunities that have made me who I am.

Finally, this journey would have not been possible without the support and encouragement of my husband **Sherif**. I am profoundly grateful for his love, generosity and thoughtful conversations. His love, his outlook on the world and his intellect are the sources of my motivation. Thank you.