Biomarker discovery for head and neck cancer

A proteomics approach

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Biomarker discovery for head and neck cancer
A proteomics approach

Biomarker identificatie voor hoofd-hals kanker
Een proteomics benadering

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 20 januari 2010 des middags te 4.15 uur

door

Barrie Martineke Visser

geboren op 25 november 1980 te Culemborg

Promotoren:	Prof. dr. A.J.R. Heck
	Prof. dr. R.H. Brakenhoff
Co-promotor:	Dr. M. Slijper

Aan Geert

“Laat ons drinken op al onze vrienden die hier niet zijn...!”♫

Pater Moeskroen, uit Zeemanslied (Steelt de schouw!, 1994)

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Chapter 1

General introduction

HEAD AND NECK SQUAMOUS CELL CARCINOMA

Epidemiology and risk factors

Head and neck cancer is a broad term that covers the heterogeneous group of cancers that arise in the upper aero- and digestive tract, including the oral cavity, the pharynx, and the larynx (see Figure 1). Over 90% of these cancers develop in the squamous cell epithelium of the mucosal linings and are therefore referred to as head and neck squamous cell carcinoma (HNSCC). In total, HNSCC accounts for 6% of all cancer cases and is the sixth cause of cancer related death.[1] In 2002, more than 600.000 new cases of HNSCC and over 300.000 deaths were reported worldwide.[1] In the Netherlands, approximately 2,500 new cases of HNSCC are diagnosed each year (www.ikcnet.nl). The median age at diagnosis is in the early 60s, whereby predominantly males are affected.[1] However, the incidence of the disease is rising amongst both young people and woman, most likely due to changing lifestyles.[2] The overall 5-year-survival rate is around 50-60% and depends mainly on the tumor stage at diagnosis.

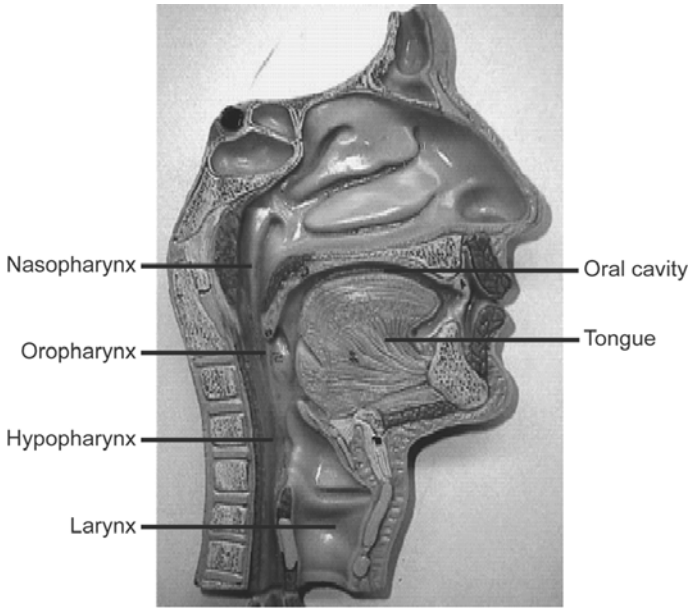

Figure 1. Upper aerodigestive tract anatomy

The major risk factors for the development of HNSCC are tobacco exposure and excessive alcohol consumption, which are both independent risk factors, but show strong synergy when combined[3-8]. By estimate, the risk increases 3 to 9 times in individuals who either smoke tobacco or consume alcohol-containing beverages, but 100 times in individuals who

combine these habits.[9] Three-quarters of all HNSCCs are estimated to be associated with excessive smoking and drinking[3], however, the causal effects of both risk factors on the various anatomical subsites are quite different[10, 11].

Besides smoking and alcohol use, infection with the sexually transmitted human papillomavirus (HPV) is at present an established risk factor, in particular for oropharyngeal cancer.[12-19] These dsDNA viruses are strictly epitheliotrophic and have a 7 kB genome with a number of overlapping open reading frames, divided in so called early and late genes. More than 100 related HPV subtypes are known of which only a subset are oncogenic and referred to as high-risk types.[20] High-risk HPVs encode two proteins, the E6 and E7 oncoproteins, that can respectively inactivate cellular p53 and Rb of the infected cells. This inactivation of p53 and Rb can initiate cellular transformation. In HNSCCs, the high-risk type HPV16 is mostly detected and involved in a distinct group of tumors that are epidemiologically and biologically different.[21-23]

Besides the classical risk factors, other habitual risk factors, that were previously localized only in non-Western countries, are spreading globally, such as chewing of tobacco, areca nut, khat leaves and mate.[24] Next to environmental carcinogens that are risk factors for HNSCC, there are also protective factors. Consumption of fruits and vegetables has shown to provide protection against HNSCC.[25, 26]

Even though lifestyle factors account for the majority of HNSCCs, genetic factors will render some individuals more sensitive for these environmental factors than others.[27, 28] A higher, genetically determined, mutagen sensitivity correlates both with a higher risk of HNSCC, and more particular with a high risk of multiple HNSCCs.[29, 30] Recent studies show that genetic variations in the enzymes responsible for the metabolism and detoxification of the carcinogenic substances in cigarette smoke and alcohol lead to variation in cancer risk.[31-33] Likewise, polymorphisms in DNA repair genes or variations in epigenetic regulation of these genes may be other causes for the variable mutagen susceptibility within the population.[34]

Thus far only one cancer susceptibility syndrome genetically predisposes to HNSCCs. Patients with Fanconi Anaemia (FA) have a very high risk to develop squamous cell carcinomas, predominantly in the oral cavity.[35] The recessive genetic disorder is caused by a biallelic mutation of one of the members of the FA/BRCA pathway.[36, 37] Cells of FA patients show chromosomal instability, particularly when treated with DNA cross-linkers, which, besides the functional studies, indicates that the pathway is involved in DNA repair.[38] The patients may show congenital abnormalities and often increasing bone marrow failure, but the phenotypes vary tremendously. They are predisposed for acute myeloid leukemia and squamous cell carcinomas. They have a 500-1,000x increased risk of squamous cell carcinoma and often develop these tumors at young age.[35]

Diagnosis and tumor classification

HNSCC is diagnosed by physical examination and microscopic examination of a tissue biopsy. HNSCCs are classified according to the TNM (Tumor, Node, Metastasis) system of the International Union Against Cancer and the American Joint Committee on Cancer, and staged by palpation, panendoscopy, and imaging tools such as CT, MRI and more recently PET.[39-42] The TNM-classification considers three clinical parameters: primary tumor size (T), the presence or absence and size of regional lymph node metastases (N) and the presence or absence of distant metastases (M) (see ref. 9 for overview). Currently, no other pathological or molecular parameters for tumor staging are considered in the TNM classification of HNSCC. Future revisions of the TNM system can be expected that will incorporate pathological and biological features of the tumor.[43] Besides the TNM system, tumors are also clinically staged to group the tumors that, despite a different TNM-classification, have a comparable prognosis. Small tumors without metastases to the lymph nodes or distant sites are classified as stage I (< 2 cm) or stage II (< 4 cm). Larger tumors or tumors with lymph node metastasis or distant metastasis are classified as stage III or IV.[9]

Treatment and prognosis

The major factor determining the prognosis of head and neck cancer is the presence or absence of lymph node metastases in the neck. Not only the presence but also the number, the level in the neck and the presence of extranodular spread are important prognostic factors. Hence, much research is focused on the accurate staging of the status of the lymph nodes in the neck. Most promising at present seems the sentinel lymph node biopsy[44-46] that enables detection of metastasis in the lymph nodes of the neck, and thereby allows an accurate staging without neck dissection.[47] Recently, it was shown that also biological properties of the tumors might have prognostic impact. Particularly the presence of HPV is associated with a more favourable prognosis.[19, 48]

The major clinical problems associated with the treatment of HNSCC patients is the development of locoregional recurrences, the development of distant metastases, and the development of second primary tumors. Treatment of recurrent disease and second primary tumors is difficult and often fails.

Treatment of HNSCC patients focuses on the primary site and the neck. Patients with stage I or stage II tumors generally receive single modality treatment, i.e. either surgery or radiotherapy, directed only at the tumor site.[49] The five-year-survival rate for this group of patients is 90%. Unfortunately, two thirds of all patients present with a stage III or IV tumor. These patients receive multi-modality treatment, consisting of surgery and radiotherapy or chemo-radiation focused on both the primary tumor and the regional lymph node

metastases.[50] Despite extensive treatment that may have major consequences for the ability to swallow and speak, and causes an impairment of the quality of life after recovery[51], the prognosis for this group of patients is much worse with a five-year-survival rate of less than 60%.

During the last decades, progress has been made in the development of improved treatment modalities for HNSCC.[49] Morbidity associated with comprehensive neck dissections was reduced by incorporation of the selective neck dissection.[52, 53] Reconstructive surgery allowed larger resections, while maintaining quality of life. Also radiotherapy improved extensively using new methods such as Intensity Modulated Radiotherapy (IMRT) and hyperfractionation. In the last decade the concurrent use of radiotherapy and cisplatin-containing chemotherapy regimens has been implemented. Although these and other developments have improved outcome, palliative care and reduced treatment-related morbidity, the five-year-survival rates have improved only moderately and still leave much to be desired.[54] Early diagnosis of primary tumors and recurrences might further improve outcome.

Early diagnosis of HNSCC

The major difference in the treatment and prognosis between early staged tumors and advanced staged tumors indicates that early diagnosis will reduce treatment-related morbidity and save lives. Early diagnosis of HNSSCs is complicated by the rapid progression of early stage squamous cancers to advanced stage, and the fact that the early stages hardly cause clinical symptoms. Although visible precursor stages might precede HNSCCs, the very large majority of tumors arise *de novo*, and are only detected once they have progressed to more advanced stages.

HNSCCs in the oral cavity might be detected by frequent thorough examination of the mouth, for which guidelines are described in ref. 9. The risk of oral cancer in the general population is relatively low, and therefore most screening studies are carried out in populations with increased risks. Only few studies have been performed to determine the efficacy of population-based screening for HNSCC by visual examination, but with contradictory results.[55, 56]. These studies have not resulted in screening programs based on visual inspection. Also the promising approaches making use of biomarkers have not resulted in large scale population-based screening programs thus far.[57] Notwithstanding, it remains an ideal approach to reduce treatment morbidity and disease mortality when more tumors were identified at stage I or II.

Recurrent cancer

A major problem in the clinical management of HNSCCs is the frequent occurrence of tumor relapses in the same or adjacent anatomical region even when the surgical margins were histologically tumor-free. Clinically these relapses are assigned as local recurrence when they develop within three years and at < 2 cm distance of the primary tumor.[58] Relapses not fulfilling these criteria are clinically classified as second primary tumors (SPT).[59, 60] Treatment of relapses is difficult as they occur in tissues affected by previous treatment.

In previous research it was shown that the high incidence of these local relapses can be explained by two different mechanisms. Local relapse may arise from unresected tumor cells that have spread locally. These residual cells remain undetected when the surgical margins are analyzed by histopathological examination, and the cells that stayed behind may develop into a local recurrence. This mechanism is referred to as minimal residual cancer.[61] The second mechanism is called “field cancerization” and describes that the tumor develops in a field of premalignant cells.[62, 63] When the primary tumor is surgically removed, a part of the premalignant field may stay behind and give rise to a new tumor that based on the clinical criteria is assigned as local recurrence or second primary tumor, while in fact the pathobiological origin is the same.

HNSCC carcinogenesis and premalignant precursor fields

As for all cancers, it has been well established that HNSCC is the result of a multistep process characterized by the accumulation of genetic and epigenetic alterations of which a fraction is already present in premalignant stages.[64] These genetic alterations, that can be detected by a variety of techniques, cause activation of oncogenes and inactivation of tumor suppressor genes through deletions, point mutations, promoter methylations or gene amplifications.[65, 66] In 1996, it was shown that the accumulation of particular genetic changes is associated with increasing histological abnormalities, and the first genetic progression model for HNSCC was presented.[67] The basis of the model, however, was still histological grading, i.e. the microscopic evaluation of the extent of abnormal appearance of the tissue morphology. A few years ago, Braakhuis *et al.*[68] described an adapted molecular progression model for HNSCC carcinogenesis. This adapted model indicates that a stem cell in a clonal unit forming the mucosal epithelium is genetically damaged. Increasing genetic damage causes that clonal unit to laterally displace a large part of the normal mucosa, and a field of genetically damaged cells develops. HNSCC arises in these premalignant fields of genetically altered epithelial cells, also called precursor or precancerous fields (Figure 2).

These precursor fields are characterized by allelic losses at chromosome arms 3p, 9p and 17p[72-74] and often show p53 pathway disruption[69-71]. The fields can reach dimensions of up to several centimeters in diameter without causing any clinical symptoms such as pain or visible changes. The large majority are not macroscopically visible, although some precursor fields appear as clinically identifiable lesions assigned as leukoplakia and erythroplakia. Very recently, it has become possible to visualize the fields using autofluorescence, which convincingly confirms their existence and dimensions.[75, 76] As indicated above, in these precursor fields a tumor can develop that will be diagnosed in the course of the disease, and is often surgically removed. As these fields are generally large and not visible to the naked eye, they are often not completely resected when a tumor is excised. Local relapses may therefore arise in the unresected fields. These local relapses are clonally related to the field and the primary tumor.[63, 71, 77-84] The identification of individuals with precancerous fields might considerably improve the early diagnosis of both primary and secondary HNSCC.

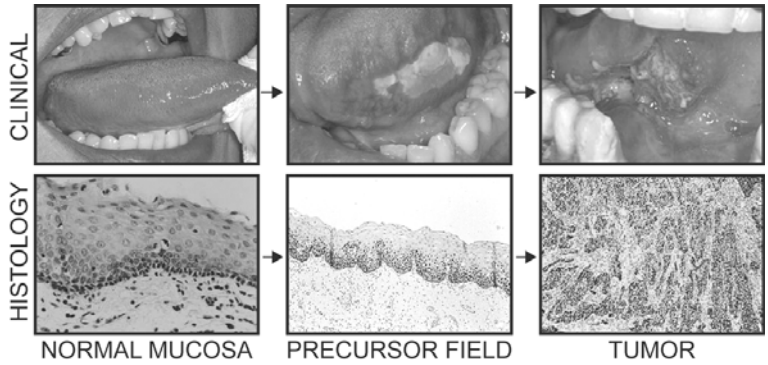

Figure 2. Simplified HNSCC carcinogenesis (available in color)

HNSCC's are preceded by precursor fields characterized by:

- allelic loss (100% of all fields)
- p53 pathway disruption (over 50% of all fields, see p53 mutation staining in histological image)
- dysplasia, various grades (the majority of all fields)
- leukoplakia (20% of all fields, see clinical image)
- no other clinical or visible symptoms (80% of all fields)

Identification and risk assessment of HNSCC premalignant fields

Dysplasia

When HNSCC patients are surgically treated, the margins of the excised specimens are routinely investigated for the presence of residual cancer and unresected premalignant fields. Most precursor fields can be recognized under the microscope as dysplastic mucosal epithelium and the presence of dysplasia in the margins of surgically removed HNSCC carries a risk of developing local recurrences.[85, 86] Grade of dysplasia is evaluated according to the standard criteria of the World Health Organization[87] as either no, mild, moderate or severe dysplasia. However, histological grading is somewhat subjective causing intra- and interobserver variability.[88] Currently only severe dysplasia in the surgical margins is considered to be a predictor of local relapse and might be an indication for post-operative radiotherapy, but never as a single criterion. Consequently, histopathological grading has limited value to predict the malignant potential in individual cases; nevertheless, due to lack of better options, it currently is the gold standard for identification and risk assessment of precursor fields in surgical margins.

Leukoplakia

Oral leukoplakias and erythroplakias are white and red plaques, respectively, that cannot be classified as any order known disorder and might carry a risk of malignant transformation.[89] These lesions represent the small subset of precursor fields that can be detected by visual inspection. The risk of malignant transformation of erythroplakias is much higher than for leukoplakias, but erythroplakias are rare, which in the light of risk assessment, caused a shift in the research focus towards leukoplakia.[90, 91] Leukoplakia has a prevalence of an estimated one to eight percent depending on age and gender and most lesions arise in the mandibular or buccal mucosa.[9] The reported malignant transformation rates vary considerably between studies depending on the clinical definitions used and follow-up time.[92] Previously it has been reported that 3-17% of all leukoplakias progress to cancer every year, with lesions at the floor of mouth possessing the highest risk.[9] More recently, this number has been adjusted to approximately 2 to 3% per year, as a result of the exclusion of lesions that were previously incorrectly defined as leukoplakia. This percentage, however, still accumulates to 50% in 16 years.[93]

The clinical management of leukoplakia currently consists of clinical inspection and biopsy followed by either excision or CO_2 laser treatment, or watchful waiting. Patients, both treated and untreated, will receive frequent (every three months) or less frequent (every 6 months) follow-up, depending on the presence of epithelial dysplasia in the biopsy.[94, 95] Histological grading of epithelial dysplasia is still the gold standard, but, as explained before, it is

somewhat subjective and therefore has limited value to predict malignant transformation in individual cases. Also the interventions targeting leukoplakia have limited effects both on the recurrence rate of the lesions as well as on the risk of malignant transformation.[96]

P53 pathway disruption

Disruption of the p53 pathway is one of the earliest events in the carcinogenesis of HNSCC, and is therefore a typical characteristic of precursor fields.[68, 97-99] The p53 pathway maintains the integrity of the cellular genome by initiating cell cycle arrest, DNA repair, cellular senescence or apoptosis in response to stress or DNA damage.[100-102] In HNSCC, the p53 pathway can be abrogated by mutation of the p53 gene, targeted degradation of the p53 protein by the HPV E6 protein[103], and probably other unknown mechanisms.[104, 105] Mutation of the p53 gene occurs in 50% of all cancers and this percentage is even higher in head and neck tumors.[104-109] The presence and type of p53 mutations in the tumors are associated with smoking habits[110] and there are some indications that the type correlates with survival[109, 111]. Missense mutations often cause over-expression of the aberrant protein, which allows detection by immunohistochemistry.[98] However, truncating mutations of p53, or enhanced degradation of the p53 protein cannot be detected by immunohistochemistry. Therefore, detection of p53 expression by immunostaining does not accurately predict the presence of a genetic change in the gene, which makes it less suitable for risk assessment.[111] Molecular analysis of surgical margins for p53 mutations on the DNA can predict local recurrence[79, 97], but the molecular analysis is extremely laborious and preferably requires freshly frozen material. This makes it less suited as diagnostic tool for the identification and risk assessment of precursor fields.

Allelic loss analysis

Allelic loss of chromosome 3p, 9p and 17p has proven to be an early characteristic of HNSCC precursor fields[86, 112, 113], and allelic loss analysis can be used for risk assessment of sites of previous oral malignancies[77, 114] or oral leukoplakias[72], and even for non-invasive diagnosis of precancerous fields by analysis of exfoliated cells[115]. Still, this methodology encounters the same obstacles for diagnostic implementation of tissue as mentioned for molecular analysis of p53 mutations. The margins need to be microdissected to enrich for precursor cells; then DNA must be isolated and analyzed by multiple microsatellite markers. The reliability of the analyses suffers when formalin-fixed paraffin-embedded specimens are used, restricting the applications. These factors hamper implementation of molecular methods in the clinic.

CLINICAL PROTEOMICS FOR PROTEIN BIOMARKER DISCOVERY

Cancer proteomics

In parallel with genomics, the term 'proteomics' is used to describe the field of research that aims at examination of the proteome, i.e. the protein complement of the genome in a certain tissue, cell type, cellular organelle or body fluid and at a certain time point.[116, 117] In the year 1996 the term 'proteome' was first coined, and also the first study on cancer proteomics was published describing the neuroblastoma proteome.[118] Protein sequence data, made available by the completion of the Human Genome Project in 2003 and the sequencing of other genomes, has enabled comprehensive protein studies.[119-121] During the recent years, proteomics technology has rapidly developed in order to take in and complement genomics approaches in biomedical settings[122, 123] and cancer research in particular[124]. A plethora of emerging methodological tools allows scientists to study the various and variable aspects of proteins, e.g. quantity, cellular location, processing, protein-complexes and post-translational modifications. Since proteins are important functional cellular molecules, proteome-based techniques hold the promise to contribute in bridging the gap between the genetic irregularities that are underlying cancer and cancer cell physiology.[125] However, the proteome's dynamic complexity requires a well considerate choice of study design, study materials, detection and identification methods, data analysis and validation studies, complemented with optimal integration between clinicians, cancer researchers, mass spectrometrists and biostatisticians.

Cancer protein biomarkers

Currently, most cancer proteomics studies are eventually aiming at the discovery of cancer biomarkers. A cancer biomarker can be any kind of biomolecule that indicates the presence or specific properties of a (pre)cancer cell.[126] Cancer biomarkers are required for diverse applications, e.g. prevention, early diagnosis, improved staging, disease-monitoring, treatment response prediction and tailored treatment .[127-129] The increased optimism for cancer biomarker discovery studies can be explained by two recent developments: the available new data resources such as the complete human genome sequence and that of the most widely used animal model, the mouse[130, 131]; and the emerging comprehensive technologies that allow parallel analysis of thousands of genes or proteins.[124] The potential of genomics and proteomics approaches for the biomarker field is enormous[132]; but the search for cancer biomarkers is still a challenging and lengthy course. Cancer biomarkers need to fulfil specific definitions and requirements.[124] The ideal biomarker, with the method to analyze it, should be sensitive, specific, cost-effective, fast and robust against situational

variability; also it should have added value beyond that of the current standards.[133] Still, it depends on the exact application which of these parameters are most crucial and which are less important.

Biomarker development follows distinct phases: an exploratory phase, a first validation phase, a phase of retrospective studies, a phase of prospective studies and finally implementation studies.[134] Especially the last two phases are time-consuming and require testable hypotheses and sufficient measurements to draw statistically valid conclusions.[135] Only few biomarker candidates have actually been approved by the US Food and Drug Administration (FDA).[136] This illustrates that the majority of candidates identified in the exploratory phase fails during the following phases. Further, this is probably also due to the fact that genome- and proteome-wide analyses deliver many possible candidates that have not (yet) been taken to the validation phases.

Compared to genomics, proteomics is a younger field of research and furthermore a more multifaceted endeavour. In general protein biomarker discovery faces more, but also some similar, hurdles when compared to the discovery of genetic markers.[123, 127] Proteins are complex, dynamic structures with a variety of possible modifications and shapes and it requires special skills and conditions to properly extract them from available patient material. With the current proteomics methodologies relatively large amounts of proteins are required that are usually not available from clinical samples. While for DNA and RNA amplification methods are at hand, these are lacking for proteins. Also protein extraction requires fresh material, which demands new logistic routes to obtain tissues as the standard pathology routine would be to fix all patient tissues in formalin. These and other limitations, such as the enormous dynamic range in concentrations of different proteins, always restrict clinical proteomics studies to a subpopulation of all proteins instead of the full proteome. A major obstacle in protein research is the necessity to identify the proteins of interest, while for genetic studies, e.g. DNA or RNA microarray approaches, the genes of interest are known beforehand. Analogous to genome approaches the real challenge for protein biomarker development starts after the discovery phase, when possible, potential candidates need to be validated thoroughly and implemented in clinical practice.

Proteins, besides RNA molecules, are the functional products of the genome and responsible for many processes in the (cancer) cell, and therefore they can provide new insights in molecular and cellular cancer biology. Furthermore, the use of proteins gives access to a collection of completely different possible detection tools that are easier to implement into routine diagnostics as compared to DNA methods. Taken together, protein biomarkers have great potential for various applications in cancer medical practice and can either be exploited alone or complement DNA or other biomarkers.[123, 127]

To this date, clinical proteomics has supplied numerous promising protein biomarker candidates and offered new insights in cellular biology on the site. Even though, until now, most candidates are only investigated until the validation and occasionally retrospective study phase, it seems that now is the time for more targeted and in-depth approaches and well-designed validation strategies.

Proteomics technology

The proteome-wide investigation of proteins requires three essential technological efforts: the separation, the identification and the quantification of multiple proteins. Ever since the development of its utility to identify proteins, mass spectrometry (MS) has played a key role in almost all proteomics studies.[137, 138] Notwithstanding the high speed and accuracy of current mass spectrometers, simultaneous confident identification of all proteins in a complex cell lysate is still impossible, which is why protein separation is demanded before MS analysis. Quantification of protein expression is necessary for comparison between samples and also demands separation of the proteins; however, it can be performed either before or after protein identification. Since no single methodology is capable of performing these analyses simultaneously, different platforms, facilitated with data integration and analysis tools, are used in concert.[139] For the experiments described in this thesis, we chose to use a combination of 2-Dimensional Differential Gel Electrophorsis (2D-DIGE) and Liquid Chromatography-tandem mass spectrometry (LC-MS/MS).

Separation of proteins by two-dimensional gel electrophoresis

A long-established, however, still widely used method for protein separation is two-dimensional gel electrophoresis (2D-GE) that makes use of two of the biophysical properties of proteins.[140] With this method proteins are first separated by iso-electric focusing (IEF) based on their isoelectric point (the pH at which the net charge of the protein is zero) and second on molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The development of gel-based immobilized pH gradient (IPG) strips has greatly facilitated the reproducibility of the first separation.[141] The protein lysate is absorbed by the IPG strip and after an electric current is applied, the proteins will migrate to the pH that corresponds to their pI. Next, the IPG strip, containing the separated proteins, is transferred on top of a polyacrylamide gel. At this stage all proteins are negatively charged by the SDS in the running buffer and application of an electrical field will cause them to migrate through the gel to the cathode and result in the separation based on the size of the proteins. Various IPG strips of different lengths and spanning different pH-ranges are available. The use of an IPG strip with a broad pH-range, such as pH 3 to 10 will provide an

overview image, while the use of narrow pH ranges spanning only one or two pH-units will allow more in-depth separation including that of low abundant proteins with a pI in that specific area. If a long (24 cm) IPG strip is used in combination with a large (20 cm) second dimension gel, thousands of proteins can be resolved in one 2D gel, although the result will also depend on the available amount of protein. Visualization of the separated proteins is routinely done by coomassie blue- or silver staining of the gel,[142, 143] after which differential protein spots can be excised and identified by MS. 2D-GE is a powerful technique to qualitatively study complex protein mixtures, however, it is limited by the time-consuming and laborious workflow, the substantial gel-to-gel variation and the lack of quantitative power.

Separation and quantification of proteins by two-dimensional differential gel electrophoresis

Two-dimensional differential gel electrophoresis (2D-DIGE) technology combines 2D separation and quantification of proteins by the use of fluorescent labels (Figure 3).[144-146] Traditional protein visualization methods used after gel separation, such as silver- or coomassie blue staining, are not suited for quantification as they possess limited linear dynamic range or lack sensitivity.[140, 147-149] 2D-DIGE makes use of fluorescent dyes that were developed to covalently label protein before in-gel separation. After labelling, the protein extracts from different samples are mixed, allowing simultaneous separation of multiple samples. Scanning the gel at the appropriate dye-specific wavelengths, will generate a gel image for each corresponding protein sample.

The broad dynamic range and high sensitivity of the fluorescent dyes enables protein quantification, even when minute quantities of protein are applied. Another advantage is the reduction of gel-to-gel variation as multiple samples can be separated in one gel. Furthermore, the development of the third fluorescent dye has created the opportunity to include an internal standard with each gel.[150] This facilitates normalisation of each individual protein spot volume based on that of the standard and allows quantitative comparison between multiple gels. Spot detection and matching algorithms have been developed and improved for the simultaneous analysis of numerous gel images.[151, 152] Software packages such as PDQuest (Bio-rad Laboratories Inc., Hercules, USA) and DeCyder (GE Healthcare, Diegem, Belgium) have implemented these algorithms and provide a semi-automated data analysis workflow that substantially reduces manual interference and thereby diminishes the introduction of bias. Further, these software programs are equipped with statistical tools that can be used to select the spots of significantly differentially expressed proteins. These protein spot can be excised from the gel, either manually or automated, and in-gel digested into peptides with trypsin before MS analysis.

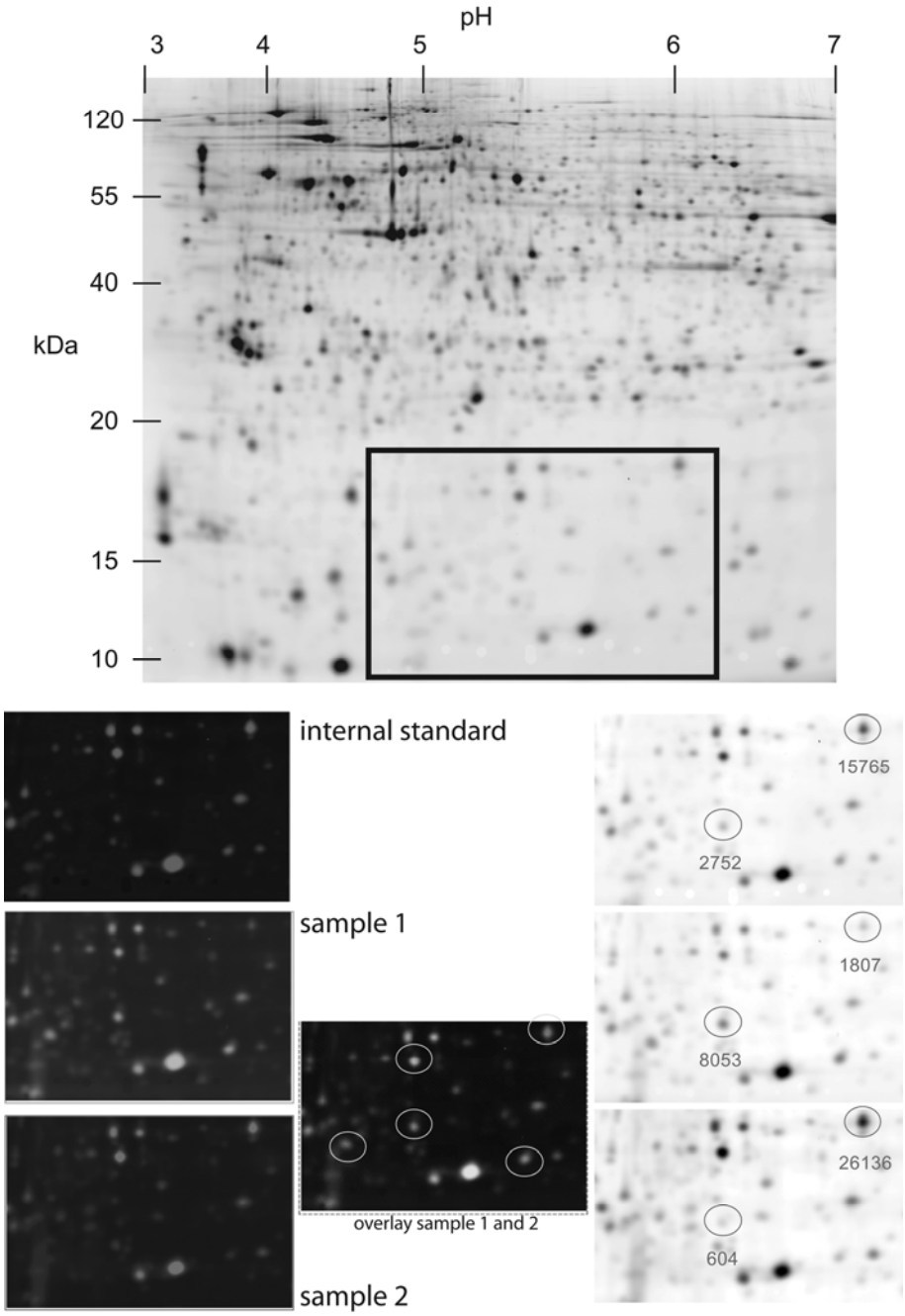

Figure 3. 2D-DIGE protein separation and quantification (available in color)

Protein samples are labelled with a fluorescent dye, mixed and separated based on pI and molecular weight. Scanning the gel at the dye-specific wavelengths will produce an image for each corresponding sample. Each spot in the image can be quantified and normalized based on the image of the internal standard.

2D-DIGE handles small protein amounts and multiple samples in a robust, straightforward and quantitative mode. The combination of these factors renders it very suitable for the proteome analysis of clinical samples.

Protein identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

In general, the mass spectrometer very accurately measures the masses of charged molecules in gas phase. The identification of proteins was enabled by the discovery that proteins or peptides can be ionised and brought into gas phase after which their masses can be measured by the mass spectrometer.[153, 154] Basically, the mass spectrometer consists of three main segments: the ion source, where the proteins or peptides are charged; de mass analyzer, that separates the molecules based on their mass relative to their charge (m/z value); and the detector, that generates a spectrum in which the intensity of the ions is plotted against the m/z value. Proteins or peptides can be ionised either from solid state by matrix-assisted laser desorption/ionization (MALDI)[154] or from liquid state by electrospray ionization (ESI)[153]. Since for identification proteins are often digested into peptides and separated by liquid chromatography (LC) before MS analysis, ESI is the current most popular method of choice. A variety of mass analyzers are used and they all have different working principles and therefore different strengths and weaknesses as reviewed in ref. 145. Nowadays, mostly more than one mass analyzers are used in parallel or simultaneously for separation and detection of the molecules in order to combine their strengths. This also allows tandem mass spectrometry (MS/MS). MS/MS is a configuration whereby a first MS determines the peptide masses, then the peptides are further fragmented, and a second MS measures the masses of the fragments. The produced MS and MS/MS spectra, respectively, provide peptide masses and partial sequence data, that can be mapped against theoretical digests of all proteins in the human database to identify the protein of interest.[155]

Alternatives for protein separation and quantification

Other more recently developed separation methods are based on different, mostly peptide, features, e.g. multidimensional LC, ion exchange chromatography, and peptide iso-electric focussing (IEF) methods.[156] Several of these methods can also be employed to specifically select for and study post-translational modifications (PTM's) such as phosphorylation.[157] Major efforts have been made in the improvement of protein quantification in order to be able to compare multiple samples. In general techniques are either label-free or label-based.[158] Label-free quantification uses the number of detected MS or MS/MS spectra to determine protein expression. This is referred to as spectral counting and requires a stable

MS system and very consistent sample handling and data acquisition; still, the accuracy drops when the changes in expression decrease or the number of samples increases.[159] More robust methods depend upon labelling of samples with stable isotopes, after which samples can be mixed and analyzed in concert allowing comparison of the ion intensity of peptides.[160] Several stable isotope labels are available of which some can be introduced during cell culturing or breeding of experimental animals and others are introduced after the protein extract is harvested. Of course the first method does not apply to human materials and also most of these labelling approaches are not very robust when analyzing multiple samples with small amounts of proteins.

Applied protein biomarker discovery workflow

In Figure 4 the biomarker discovery workflow followed for the studies described in this thesis is depicted. We used both a p53 inactivated cell model and patient tissues for proteome analysis. With the p53 inactivated cell model we specifically set out to find mutated p53-related biomarkers. For the tissue analysis we first performed genetic characterization to ensure truly normal and precursor tissue. To be able to compare multiple clinical samples with low protein amount, we decided to use 2D-DIGE as a quantitative method. Both PDQuest and DeCyder software packages were used to match and analyze the different gel images. After trypsin digestion of the differential protein spots, proteins were identified by LC-MS/MS. Evaluation and further validation of the most promising protein biomarkers was performed by immunohistochemistry on the cell model and different tissues of relevant patient cohorts.

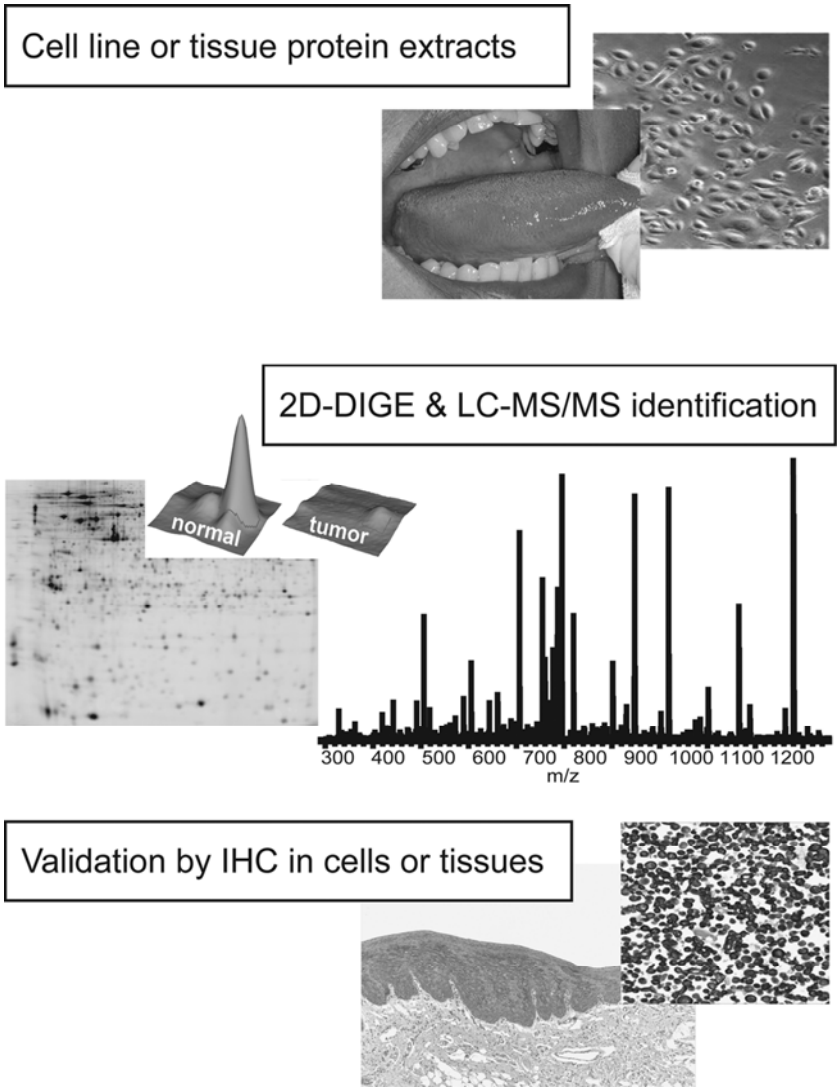

Figure 4. Protein biomarker discovery workflow

AIM AND OUTLINE OF THIS THESIS

The availability of protein biomarkers for identification of progressing precursor fields for HNSCC would permit screening of the surgical margins of treated patients by standardized immunohistochemical methods. This could easily be implemented into the daily practice of diagnostic laboratories and thereby significantly improve the early detection of local relapses in the same or adjacent anatomical region. Further, such protein biomarkers might be used to discriminate progressing leukoplakias from those that do not progress. Other applications could be the identification and grading of precursor fields using brushed cells or saliva, analogous to the detection of genetic alterations in brushed cells or methylated DNA in saliva.[161] However, at this moment no such protein biomarkers are available.

In brief, the studies described in this thesis were aimed at discovery, validation and initial retrospective evaluation of protein biomarkers for the early detection of HNSCC with a specific focus on precursor fields. For discovery and validation we first used a cell model and second a series of actual patient tissues. To evaluate the potential biomarkers for clinical use we performed retrospective, prognostic studies with leukoplakia samples and surgical margins of treated HNSCC patients.

In Chapter 2 it is described how we employed a conditionally immortalized keratinocyte cell model to mimic p53 inactivation similar to p53 pathway disruption in HNSCC precursor fields. This model enabled the comparison between proteomes of cells with different types of p53 pathway inactivation and proteomes of normal, primary keratinocytes. We aimed at discovering markers for p53 pathway interruption irrespective of the type of p53 inactivation. Potential protein candidates were verified by immunostaining on cells and precursor field tissue.

For the study in **Chapter 3** genetically characterized and paired normal, precursor and HNSCC tissues were compared on the proteome level. The paired sample design allowed more sensitive statistical analyses, which provided promising candidate biomarkers. Immunohistochemistry was used to validate differential protein expression in the same and in an independent tissue sample set.

In **Chapter 4** is described whether the most promising protein biomarkers identified Chapter 3 could be exploited to detect leukoplakias at risk of malignant transformation. In a retrospective setting, the expression of several candidates was analyzed by semi-quantitative immunohistochemistry in a series of progressing and non-progressing leukoplakias.

In **Chapter 5** was evaluated, by a retrospective case-control study, if the potential biomarkers were suitable to predict the development of local relapse by the immunostaining

of the resection margins of surgically treated head and neck cancer patients. Retrospectively, all surgical margins were stained of a cohort of patients in order to find a relationship with local relapse-free survival time.

In **Chapter 6** an overview is provided of many of the reported proteomics studies performed in the field of head and neck cancer in the past few years. In this review we describe the shift in clinical issues and summarize the various approaches that have been developed to characterize HNSCC carcinogenesis on the proteome level, and to provide new candidate biomarkers for screening, staging, prediction of prognosis and treatment response. Therewith, this Chapter offers a broader perspective for the work described in this thesis.

The final **Chapter 7** aims to summarize and discuss the results in general and give future recommendations for the analysis of HNSCC protein biomarkers.

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Chapter 2

Comparative proteome analysis to explore p53 pathway disruption in head and neck carcinogenesis

Tieneke B.M. Schaaij-Visser
Ruud H. Brakenhoff
Jeroen W.A. Jansen
Martina C. O'Flaherty
Serge J. Smeets
Albert J.R. Heck
Monique Slijper

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ABSTRACT

The 5-year-survival rate of head and neck squamous cell carcinoma (HNSCC) has been only moderately improved over the last few decades. HNSCC develops in precursor fields of genetically altered mucosal cells, typically characterized by p53 pathway disruption, that mostly do not give any clinical symptoms. Patients present therefore often with invasive carcinomas in an advanced stage. After tumor resection, part of these fields frequently stay behind unnoticed, causing secondary tumors. Identification of these precursor fields would allow screening and early detection of both primary and secondary tumors.

Our aim was to identify differential proteins related to p53 dysfunction. These proteins may serve as valuable biomarkers that can predict the presence of a precursor field. We used a squamous cell model for p53 inactivation, which was analyzed by 2D-DIGE and LC-MS/MS. This approach enabled us to identify a set of 74 proteins that were differentially expressed in cells with normal versus disrupted p53 function. For six proteins the major changes in expression were verified with immunohistochemical staining. The most promising result was the identification of peroxiredoxin-1 which allowed immunohistochemical discrimination between normal epithelium and precursor field tissue with a *TP53* mutation.

INTRODUCTION

Head and neck cancer develops in the mucosal linings of the upper aero- and digestive tract and is the sixth most common cancer worldwide.[1] More than 90% of head and neck tumors are squamous cell carcinomas. Specific risk factors for developing HNSCC are tobacco smoke, alcohol abuse, and infection with the human papilloma virus (HPV).[2] Currently, the overall five-year-survival rate of HNSCC is approximately 60%.[1] A large proportion of patients presents with advanced stage disease and despite combined and improved treatment modalities, the prognosis still leaves much to be desired.

Braakhuis *et al.*[3] have recently described an adapted molecular progression model for the carcinogenesis of HNSCC. This model indicates that head and neck tumors develop in a large precursor field of genetically altered epithelial cells.[4-6] Although these fields can reach dimensions of multiple centimeters in diameter, they mostly do not elicit clinical symptoms. Only approximately 20% of the fields can be detected as leukoplakia or erythroplakia, i.e. as white or red patches on the mucosa, respectively. Since the precursor fields are larger than the primary tumor and mostly invisible, they are not always completely resected when the tumor is excised. The remaining field can lead to the development of secondary tumors, clinically assigned as local recurrences and second primary tumors.[6, 7] The availability of biomarkers to identify these precursor fields would thus permit analysis of the surgical margins for fields, and additionally allow more frequent surveillance during follow-up and early diagnosis of second primary tumors. Although precursor fields can be identified by genetic analyses, these methods are laborious, necessitating micro-dissection of tissue biopsies, and of limited sensitivity. Detection of these fields by immunohistochemistry using suitable protein biomarkers would facilitate implementation of this knowledge in clinical management.

Disruption of the p53 pathway is one of the earliest events in the carcinogenesis of HNSCC, and is therefore a typical characteristic of precursor fields.[8, 9] The p53 pathway maintains the integrity of the cellular genome and is composed of a network of proteins that responds to a variety of stress signals. This network involves cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation, and cell division.[10] Stress signals induce site-specific post-translational modifications of p53, like protein serine- and threonine phosphorylation, and lysine side chain acetylation, -methylation, -ubiquitination or -sumoylation. This results in activation of p53 as transcription factor, initiating cell cycle arrest, DNA repair, cellular senescence or apoptosis.[11] A number of feed-back loops have been described that act upon the p53 response, of which many act through the MDM-2 protein that targets p53 for proteasome-mediated degradation. A well-known stress signal

that activates the p53 pathway is DNA damage, although the different types of DNA damage activate distinctive enzyme activities, for example gamma irradiation activates ATM kinase and CHK-2 kinase, whereas UV radiation activates ATR, CHK-1 and casein kinase-2. More than 4800 genes carry one or more p53 binding motifs.[12], and it is thought that specific gene regulation is related to both cell type and stress type. This makes p53 one of the most prominent tumor suppressor proteins.

In HNSCC, the p53 pathway can be abrogated by mutation of the p53 gene, targeted degradation of the p53 protein by the HPV E6 protein, and inactivation of any of the modulators of p53.[13] Mutation of the p53 gene occurs in 50% of all cancers and this percentage is even higher in head and neck tumors.[13, 14] Different types of p53 mutations were found with distinct effects on p53 protein expression. Most p53 mutations found in human cancers are missense mutations that result in over-expression of an altered p53 protein with dominant-negative activity.[14] Over-expression of the aberrant protein allows detection by IHC.[9] In contrast, nonsense or frameshift mutations generate truncated or unstable p53 proteins that subsequently are degraded and cannot be detected by IHC. The same holds true in the case of HPV E6 induced degradation of p53. These various effects of p53 pathway disruption on the protein level hamper the application of p53 as a protein biomarker. Since p53 function loss is typically one of the earliest changes in carcinogenesis[3], proteins that indicate p53 dysfunction may act as valuable biomarkers to identify precursor field presence, however, no such markers are available.

The aim of this study was to detect protein biomarkers that indicate p53 malfunction, by using a quantitative proteomics approach. To investigate the effect of p53 function disruption, we used a conditionally immortalized squamous cell model in which the described modes of p53 inactivation were introduced, i.e. missense and nonsense mutations and inactivation by HPV E6. These cell models for p53 pathway inactivation were compared with wild type p53 control cells by differential proteome analysis through 2D-DIGE and LC-MS/MS, to determine which protein levels and pathways are affected. 2D-DIGE was chosen as it allows relative quantification of protein levels for many samples simultaneously by the use of an internal standard. Moreover, 2D-DIGE permits evaluation of proteins at a wide range of expression levels, due to the 4 log dynamic range of detection the CyDyes. Immunohistochemistry was used to verify the results on the cell model and to assess the value of the detected proteins as potential biomarkers to detect precursor field tissue.

MATERIALS AND METHODS

Figure 1 represents the schematic workflow, showing the preparation steps to create the p53 inactivation model. This was followed by 2D-DIGE analysis of the lysates, protein identification by LC-MS/MS and verification of the results using immunohistochemistry.

Conditionally immortalized human squamous cell model

The generation of this model for p53 inactivation and its characteristics are described elsewhere.[15] In short, primary keratinocytes were harvested from surgically removed uvulas of patients treated for snoring. To create the conditionally immortalized squamous cell model, the temperature-sensitive SV40 large T antigen gene (tsLT) and the catalytic subunit of telomerase (hTERT) were introduced into the primary keratinocytes, as described previously for hepatocytes and fibroblasts.[16, 17] The tsLT blocks p53 and pRb at 32ºC, allowing the cells to proliferate at the permissive temperature of 32ºC. At 39ºC, the tsLT is inactive, and the cells immediately enter proliferation arrest in less than one population doubling. The cells die after this proliferation arrest within 1-3 weeks after temperature shift to 39ºC, most likely by apoptosis and similar to normal primary keratinocytes at the end of their lifespan.[15]

Inactivation of p53 in the squamous cell model

To mimic p53 inactivation in tumors, the tsLT transfected cells were transduced with either one of the following constructs: 1) dominant negative mutant p53 (R175H) (dn p53; mimicking missense mutation), 2) short hairpin p53 RNA (shR p53; mimicking nonsense mutation) [16], and 3) the HPV E6 gene (mimicking HPV infection). Clones were obtained from Prof. R. Bernards and Dr. K. Berns, Netherlands Cancer Institute, Amsterdam, and Dr. E. Hooijberg, Pathology, VUMC, Amsterdam, The Netherlands. Transduction was performed with the retroviral Phoenix cell system according to Smeets *et al.*.[15] After transduction at 32ºC, the cells were shifted to 39ºC. In every experiment both untransfected cells and green-fluorescent protein-transfected cells were used as controls for transfection. As indicated above, without p53 abrogation these cells enter an immediate proliferation arrest at 39°C and die within 1-3 weeks. After p53 inactivation the cells show an extended life span of 15 population doublings.[15] Three different primary keratinocyte cell cultures, expanded at 39ºC, were used as normal reference. The tsLT conditionally immortalized keratinocytes, either not transduced or mock transduced, could not be used as normal reference, since these cells would immediately stop proliferation and go into apoptosis when shifted to 39°C.

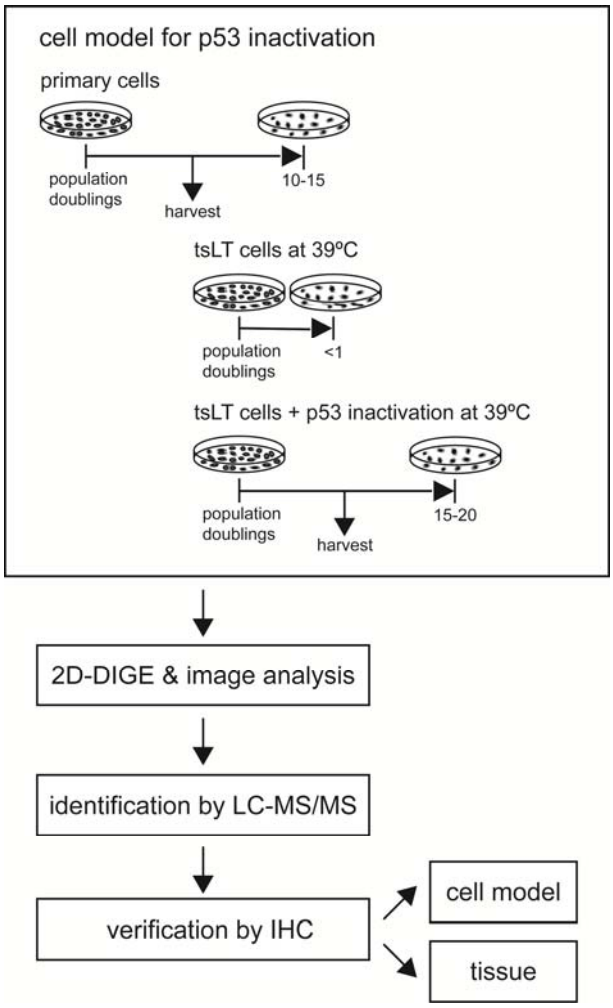

Figure 1. Schematic workflow

To create a conditionally immortalized squamous cell model, a temperature-sensitive SV40 large T (tsLT) antigen gene was introduced into primary keratinocytes, which renders the cells immortal when grown at 32ºC. When shifting to 39ºC, tsLT is inactive and the cells show an immediate proliferation arrest (within one population doubling) and die within 1-3 weeks, a premature senescence phenotype identical to primary keratinocytes at the end of their normal lifespan. Cells were transduced with p53 inactivating genes, 1) dominant negative mutant p53 (R175H) (missense mutation mimic), 2) short hairpin RNA of p53 (nonsense mutation mimic), or 3) HPV16 E6 viral oncogene (HPV infection mimic). Transduction was performed at 32ºC, and the incubation temperature was subsequently shifted to 39ºC. The inactivation of p53 allows the cells to extend their lifespan with approximately 15-20 population doublings. Proteomes of the p53 inactivated cells and control cells were compared by 2D-DIGE, and proteins of interest were identified with LC-MS/MS. Verification was performed with IHC on both the squamous cell model and patient tissues.

The p53 inactivated cell models and primary keratinocytes were harvested while still in logarithmic growth phase, 5-10 population doublings after temperature shift or inoculation, respectively. For each p53 inactivation type, 10 µg of the protein lysate was used for Western blotting in order to analyze the p53 levels, and also p21 protein levels to detect remaining p53 activity. Antibodies were obtained from DAKO (Glostrup, Denmark) for p53, from Calbiochem (Darmstadt, Germany) for p21, and from Abcam (Cambridge, UK) for actin, and applied in dilutions of 1:1000, 1:200, and 1:1000, respectively. Secondary antibody was Cy5-labeled goat-anti-mouse antibody (GE Healthcare); dilution 1:2500). Blots were scanned using a Typhoon 9400 Imager (GE Healthcare, Diegem, Belgium) according to the instructions of the manufacturer.

Sample preparation and labeling

Adherent cultured cells were harvested on ice by scraping in lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40, one tablet complete-mini-protease-inhibitor-cocktail/25 ml [Roche, Basel, Switzerland]). To ensure proper lysis with maximum protein yield, cell extracts were homogenized with a Dounce homogenizer. Protein lysates were cleaned from other cellular molecules and buffer salts (Plus One 2D Clean-up kit; GE Healthcare) and resuspended in labeling buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS, pH 8.5), and protein content was determined (Plus One 2D Quant kit; GE Healthcare).

Protein samples were labeled with CyDye DIGE Fluor minimal dyes (GE Healthcare) as described by Kolkman *et al.*.[18] Briefly, 50 µg of protein sample was incubated with 400 pmol of Cy3 or Cy5 for 30 minutes and the reaction was quenched by adding 1 µl of a 10 mM lysine solution. An equal mixture of all samples was used as internal standard (IS) and labeled with Cy2. One p53 inactivated sample (Cy5 labeled), one control sample (Cy3 labeled) and one internal standard (Cy2 labeled) were mixed, snap-frozen and stored at -80˚C until further use.

2D-DIGE

Two dimensional separation of the protein extracts was performed as described before.[18] In the first dimension, proteins were resolved by IEF using the following pH-gradients/strip lengths: 3-7 (non-linear)/24 cm, 6-11 (linear)/18 cm and 3-10 (linear)/24 cm (GE Healthcare). For the second dimension, 12.5% polyacrylamide gels were used. Gel images were obtained with the Typhoon 9400 Imager according to the instructions of the manufacturer. PDQuest software (version 7.4.0, Bio-Rad Laboratories Inc., Hercules, USA) was employed to analyze the gel images. The protein spots were first detected in all gel images, and subsequently matched between all images. The spot volumes were normalized

according to the internal standard. The three different types of p53 inactivation that stand for 1) p53 missense mutation, 2) p53 nonsense mutation, and 3) p53 degradation through HPV E6 expression, were together considered as the test group that was evaluated against the three different primary keratinocyte cell cultures as control group. The average spot volumes of the p53 inactivated samples were compared to the corresponding average spot volumes of the control samples. Proteins were considered differentially expressed if either the average spot intensities showed a significant difference (Student's t-test, FDR-corrected $p \leq 0.05$) between the test group and the control group or if the average spot intensities were at least 4-fold up- or downregulated between the two groups. Furthermore, proteins in spots that were uniquely detected in either the test group or the control group, were also considered to be of interest. When testing hundreds of proteins for statistical significance with Student's t-test wihle only a limited sample size is used, many of these proteins may have achieved a significant p-value by chance alone. Therefore, to correct for false discovery, p-values were adjusted using Benjamini-Hochberg algorithms, which was performed with the p.adjust function from the Bioconductor 'stats' package, executed in R version 2.6.2.

In-gel tryptic digestion and protein identification by LC-MS/MS

Protein spots to be identified were picked from the analytical gels (in total 150 µg protein). A full scale Cy2 image was printed and positioned under the analytical gels to enable manual excision of protein spots of interest, since protein staining can give adverse effects with respect to MS-analysis. Afterwards, gels were scanned again to ensure correct spot excision. In-gel tryptic digestion was carried-out on the gel plugs according to the protocol by Shevchenko *et al.*.[19]

Nanoscale LC-MS/MS was performed by coupling an Agilent 1100 Series LC system to a LTQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA) as described by Scholten *et al.*.[20] Peptide mixtures were concentrated and desalted using an on-line C18 trap column (OD 375 µm, ID 100 µm packed with 20 mm of 5 µm AQUA C18, RP particles (Phenomenex, Torrance, USA)) and further separation was achieved by gradient elution of the peptides onto a C18 reverse phase column (OD 375 µm, ID 50 µm packed with 15 cm of 3 µm C18, RP particles (Reprosil)). MS analysis in the LTQ mass spectrometer was achieved by directly spraying the column eluent into the electrospray ionization source of the mass spectrometer via a butt-connected nano-electrospray ionization emitter (New Objective, Inc, Woburn, USA). The total analysis time was 1 h and mobile phase buffers were: A, 0.1 M acetic acid; B, 80% acetonitrile/ 0.1 M acetic acid. A linear 40 min gradient (10-45% B) was applied for peptide elution into the MS at a final flow rate of 100 nl/min. The

LTQ was operated in positive ion mode, and peptides were fragmented in data-dependent mode.

All MS/MS files were merged into a single file that was used as input for the MASCOT search engine (version 2.1.02, Matrix Science, London, U.K.). Spectra were searched against the National Center for Biotechnology Information non-redundant database (NCBInr, date 20060303) with *Homo sapiens* selected as taxonomy (140,507 sequences). Further search settings were: trypsin digestion with maximal two missed cleavages, carbamidomethyl (C) and oxidation (M) as fixed and variable modification, respectively, peptide tolerance of 0.5 Da with 1+, 2+ and 3+ peptide charges and MS/MS tolerance of 1 Da. All protein spots were excised and analyzed from at least two different analytical gels.

Identified proteins were further analyzed using the software program BiNGO[21] to assess the overrepresentation of Gene Ontology 'molecular function' categories of these proteins against the human proteins database. A statistical hypergeometric test was performed to determine if one or more of these GO-categories were overrepresented, and the p-value was FDR-adjusted through Benjamini-Hochberg correction. The chosen significance level was 0.05.

Immunohistochemical verification of DIGE data

For immunohistochemistry, cell pellets of each of the separate p53 inactivated cell lines and the normal control primary keratinocytes were fixed in 4% paraformaldehyde for 4 h at room temperature or overnight at 4ºC. Subsequently, the fixed cell pellets were embedded in paraffin with the Shandon Cytoblock Cell block system (Thermo Fisher Scientific, Inc., Waltham, USA) according to the instructions of the manufacturer. Furthermore, two FFPE normal mucosa tissues were selected for IHC analysis, together with two FFPE margins from surgical specimens that stained positive for p53 (precursor fields), and for which the mutations were confirmed by sequencing.[9]

The anti-peroxiredoxin-1 antibody was from Abcam (Cambridge, UK). MnSOD antibody was a gift from Prof. J.J. Cullen (University of Iowa Hospitals and Clinics, Iowa City, USA); lactoglutathione antibody was a gift from Dr. B. Kuhla, (IZKF Leipzig, Leipzig, Germany); FK506-BP5 antibody was a gift from Dr. F.M. Romano (Federico II University, Naples, Italy); the keratin 5 and -14 antibodies were a gift from Ing. T.M. Tadema, and Ing. W. Vos (VU University Medical Centre, Amsterdam, the Netherlands). Primary rabbit polyclonal antibodies were used for peroxiredoxin-1 (1:400) and manganese-containing superoxide dismutase (1:3000), primary goat polyclonal antibody for FK506-BP5 binding protein 5 (1:400), and primary mouse monoclonal antibody for lactoylglutathione lyase (1:20) and

keratin 5 (1:20) and keratin 14 (1:200). All other antibodies were from DAKO, Glostrup, Denmark.

Five µm sections were cut and deparaffinized. Optimization was performed for every step of the staining protocol and for each antibody separately. Antigen retrieval was performed by either one of the following methods: microwave boiling in Tris/EDTA-buffer (10 mM Tris pH 9.0, 1 mM EDTA) was applied for keratin 14 staining, 30-minute pepsin treatment (0.1 % pepsin in 0.02 M HCl) was applied for keratin 7 staining, and microwave boiling in citrate-buffer (10 mM citrate pH 6.0) was used for all other antibodies. For mouse and rabbit primary antibodies, normal goat serum (1:20) was used to pre-incubate prior to the 1-hour incubation step with the primary antibody at room temperature. The next step was performed with a mixture of biotinylated goat-α-mouse and goat-α-rabbit secondary antibodies. For goat primary antibodies, pre-incubation was done with normal rabbit serum (1:50) and biotin-conjugated rabbit-α-goat secondary antibodies (1:500) were used for the next step. In all cases, the final incubation was carried-out with streptavidin-biotin-complex labeled with horseradish peroxidase (StreptABComplex/HRP) and diaminobenzidine with H_2O_2 was used as a chromogen. Sections were counterstained with haematoxylin and cover slipped with Kaiser's glycerin. Irrelevant IgG antibodies from the host animal species of the primary antibody were used as negative control. P53 staining (DO-7 antibody, 1:500) was used as a positive control for mutated p53 mucosa (precursor field) as described by van Houten *et al.*. [9] Microscopic evaluation of the stained sections was performed by two independent observers.

RESULTS

Cell model for p53 function disruption

As shown in Figure 1, the introduction of the temperature-sensitive SV40 large T antigen gene into primary keratinocytes renders them immortal at 32ºC, as they were able to reac h over 100 population doublings. However, at 39ºC the SV40 large T antigen is inactive, resulting in an immediate proliferation arrest, which is a premature senescence phenotype identical to primary keratinocytes at the end of their lifespan. To mimic the p53 pathway inactivations that are observed in tumors and precursor fields of patients, we transduced the tsLT immortalized keratinocyte cells with the following constructs: 1) the dominant negative p53 mutant gene, 2) short hairpin p53 RNA, or 3) the HPV E6 gene, and shifted the culture to 39ºC. These transductions enabled the cells to extend their lifespan for approximately 15-20 population doublings. The Western blot in Figure 2 shows that p53 levels were reduced, while the levels were relatively high when over-expressed as dominant negative mutant.

Decrease of p53 activity was detected by the reduced levels of p21 for all three p53-inactivated keratinocyte cell lines.[22]

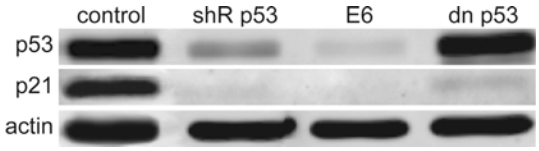

Figure 2. Western blot verification of p53 inactivation

Verification of p53 inactivation in the squamous cell models, shown by p53 and p21 levels in the various manipulated tsLT cells. Actin was used as loading control. SV40 large T antigen binds p53 but also stabilizes the protein, thereby providing a suitable positive control.[22] For shR p53 and E6 inactivation a clear downregulation of p53 and the downstream protein p21 are seen. For the dn p53 inactivation model, expression of the dominant mutant protein is detected, however, the decreased level of p21 expression indicates that this p53 protein is not active.

2D-DIGE and identification by mass spectrometry

Figure 3A depicts typical examples of 2D-DIGE gel images of the internal standard, separated in the first dimension with pH-ranges 3-7 (non-linear) and 6-11 (linear) (2D-DIGE gel image of pH-range 3-10 is not shown). Quantitative analysis of the gel images was carried-out with PDQuest software. On average 1,200 spots were detected on the pH 3-7 gel images, 600 spots on the pH 6-11 gel images and 1,400 on the pH 3-10 gel images. In total, 1,600 different spots could be matched for all gels and relatively quantified. For differential analysis, all three p53 function disruption types were considered as one group, the test group, and the primary keratinocytes as the control group. Altogether, 77 spots were detected in these gel images with differential protein levels between the test group and control group. A number of these differential protein spots were detected in more than one pH-range. For these protein spots, a clear correlation was found between the expression levels as quantified from the gel images from different pH-ranges, which corroborated our results (correlation coefficient = 0.86). Increased intensities were detected for 40 unique spots, and decreased intensities for 37 unique spots, indicating up- or downregulation of corresponding protein expression due to p53 inactivation, respectively. After excision and digestion of the spots of interest, LC-MS/MS was employed to identify the corresponding proteins. Table 1 lists the identified proteins, and Figure 3B illustrates examples of detected spots of both the upregulated peroxiredoxin-1 and the downregulated glycerol-3-phosphate dehydrogenase. Proteins from eleven spots could not be identified by MS due to low levels of expression and/or contamination of a nearby situated spot of a highly abundant protein.

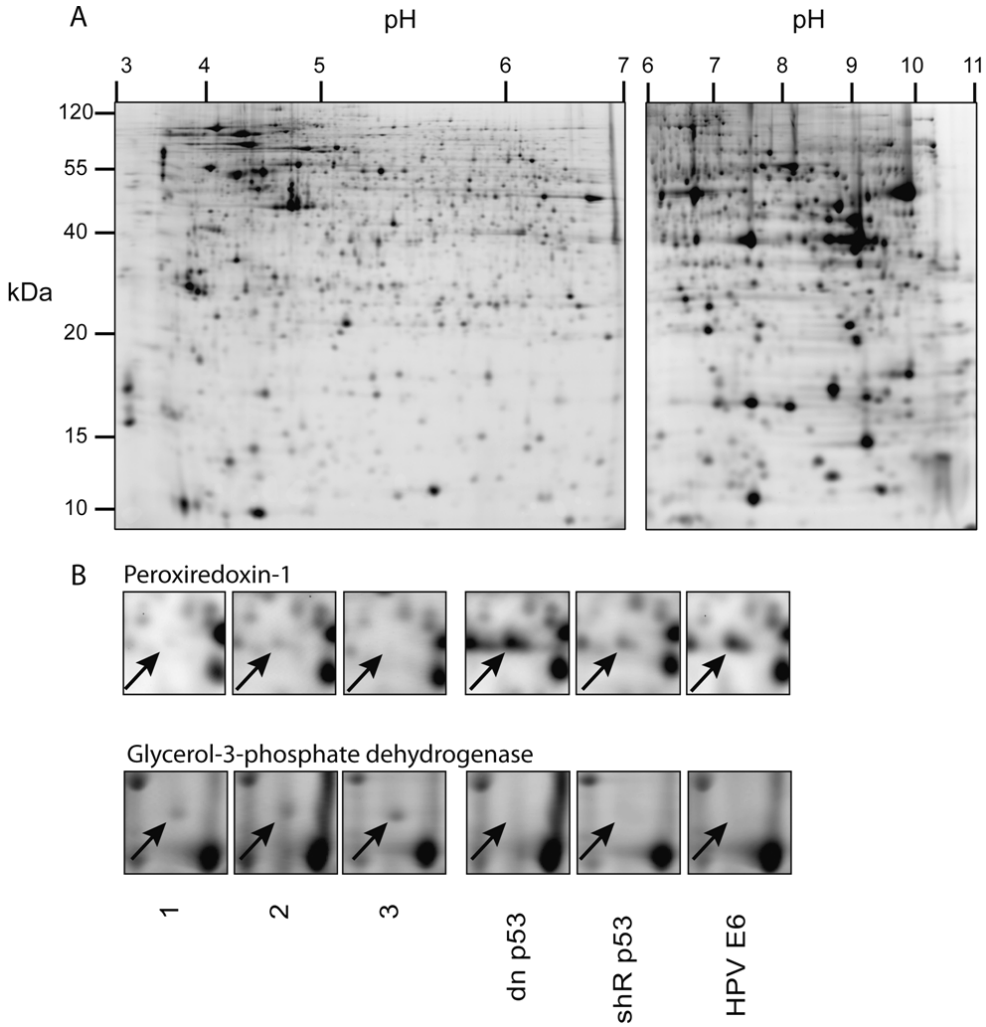

Figure 3. 2D-DIGE analysis

A. Typical examples of pH 3-7 and pH 6-11 gel images of the Cy2-labeled pooled internal standard. B. Differential expression of peroxiredoxin-1 and of glycerol-3-phosphate dehydrogenase (not normalized); 1, 2, and 3 are three different control samples; dn p53 (dominant negative p53 gene), shR p53 (short hairpin RNA) and HPV E6 (HPV E6 gene) are the three different lysates of cells with inactivated p53.

Table 1. Differentially expressed proteins

protein name	*accession number*	*fold change*	*p-value*	*controls ± stdev*	*p53 negative ± stdev*	*# peptides; kD; coverage (%)*
Keratin, type II cytoskeletal 7	P08729	>30	0.175	0	633±602	20;51;38
Peroxiredoxin-1	Q06830	31.3	0.101	75±91	2337±1538	3;22;16
Ubiquitin carboxyl-terminal hydrolase isozyme L1*	P09936	16.0 18.3	0.216 0.133	67±25 270±157	1079±1101 4948±3846	12;25;53
Activator 1 37 kDa subunit	P35249	15.4	0.023	112±193	1712±549	13;33;42
not identified		14.4	0.106	122±96	1755±1155	
Manganese-containing superoxide dismutase	Q4ZJI1	11.9	0.068	5526±2549	65972±33798	4;25;21
Lactoylglutathione lyase	Q04760	9.7	0.095	2577±2411	24995±14543	7;21;42
SH3 domain-binding glutamic acid-rich-like 3	Q9H299	8.9	0.135	1388±191	12290±9132	5;10;43
not identified		8.4	0.286	620±240	5236±6389	
Thioredoxin-like protein 2[#] V-crk sarcoma virus CT10 oncogene homolog	O76003 Q96HJ0	7.1	0.012	169±17	1201±269	9;33;28 6;34;19
not identified		7.0	0.251	669±442	4692±5068	
FK506-binding protein 5	Q13451	7.0	0.032	404±218	2821±973	27;51;50
Transgelin-3 (langer in Mascot)	Q9UI15	6.0	0.101	437±32	2618±1482	8;22;45
Beta-hexosaminidase alpha chain[#] Nicotinamide N-methyltransferase	P06865 P40261	5.4	0.106	861±136	4684±2698	9;64;13 8;30;30
not identified		4.4	0.125	410±132	1814±1104	
Phosphoglycerate kinase 1	P00558	4.4	0.135	586±209	2584±1655	12;45;27
Collagen-binding protein 2	P50454	4.4	0.113	1043±303	4573±2584	11;47;29
Stathmin	P16949	4.4	0.045	6735±1282	29418±10365	10;17;52
not identified		4.3	0.059	196±171	849±288	
Reticulocalbin-1	Q15293	4.0	0.079	357±125	1429±634	7;39;18
Vacuolar protein sorting 29*	Q9UBQ0	2.5 3.8	0.027 0.012	459±225 266±114	1148±144 1011±152	4;20;21
not identified		3.7	0.002	1407±239	5164±227	
Small nuclear ribonucleoprotein F	P62306	3.6	0.019	618±174	2206±473	4;10;19
TBCA protein	Q6FGD7	3.2	0.002	2931±440	9488±448	12;13;70
Profilin-2	P35080	3.0	0.020	462±217	1407±230	3;15;21
Calcyclin-binding protein[†]	Q9HB71	2.8	0.006	359±77	989±82	6;26;18
Succinate dehydrogenase iron-sulfur protein[#]	P21912	2.7	0.003	981±36	2693±237	13;30;44
Heterogeneous nuclear ribonucleoprotein K	P61978	2.6	0.002	747±107	1958±75	23;51;46
Eukaryotic translation initiation factor 4H	Q15056	2.6	0.020	614±180	1588±269	4;27;13
Beta-2-microglobulin*	P61769	2.2 2.5	0.058 0.022	1892±511 1589±340	4239±1070 3980±765	5;12;45
Ferritin heavy chain	P02794	2.3	0.011	1936±482	4401±344	6;22;26
Transaldolase	P37837	2.3	0.023	649±205	1464±213	18;37;44
Calcyclin-binding protein[†]	Q9HB71	2.2	0.013	3945±1301	8862±430	10;26;27
Histidine triad nucleotide-binding protein 1	P49773	2.2	0.007	550±65	1223±115	3;14;19
Alpha-enolase	P06733	2.1	0.022	2790±410	5962±1025	14;47;52
U2 small nuclear ribonucleoprotein B	P08579	2.1	0.023	1968±429	4168±690	3;25;13
Sorcin	P30626	2.1	0.003	1485±86	3112±234	9;20;51
Protein DJ-1	Q99497	2.1	0.003	11022±1268	22918±764	12;20;47
Proteasome subunit beta type 2	P49721	2.0	0.008	2705±439	5520±386	6;23;27
Sorting nexin-6	Q9UNH7	1.9	0.019	460±40	874±125	21;47;44
Aspartate aminotransferase[#] Developmentally-regulated GTP-binding protein 1	P00505 Q9Y295	-1.8	0.012	2406±138	1317±256	12;47;26 6;41;19
Calpactin I light chain[‡]	P60903	-1.8	0.009	95995±9696	52021±1210	8;11;59
Testin isoform 1 variant	Q53GU1	-2.1	0.002	3438±102	1638±145	28;48;45
UTP--glucose-1-phosphate uridylyltransferase 2	Q16851	-2.2	0.023	3075±359	1409±501	26;57;45
ATP-citrate synthase	P53396	-2.8	0.020	2615±179	929±520	32;121;28
Annexin A2	P07355	-3.3	0.007	30456±2912	9256±3030	24;39;58

protein name	accession number	fold change	p-value	controls ± stdev	p53 negative ± stdev	# peptides; kD; coverage(%)
Integrin alpha-6[#]	P23229	-4.0	0.124	730±426	181±60	17;119;18
Ubiquitin-activating enzyme E1	P22314					22;118;19
S100 calcium-binding protein A16	Q96FQ6	-4.2	0.022	7650±1877	1809±747	7;12;48
Calpactin I light chain[‡]	P60903	-4.6	0.192	8716±6833	1898±550	3;11;15
not identified		-4.8	0.013	1072±18	228±239	
S100 calcium-binding protein A2	P29034	-5.3	0.003	136002±11252	25468±10452	8;11;40
Aconitase	Q71UF1	-5.4	0.078	1366±653	251±124	14;86;22
Hemoglobin alpha subunit	P69905	-5.9	0.053	6550±2538	1111±759	3;69;23
not identified		-5.9	0.011	1402±241	235±131	
not identified		-6.3	0.068	865±382	137±126	
Annexin A3	P12429	-6.8	0.110	5838±3590	889±208	18;36;45
Glyceraldehyde-3-phosphate dehydrogenase	P04406	-6.8	0.059	8385±3597	1241±809	8;36;24
Keratintype I cytoskeletal 14	P02533	-6.8	0.150	14480±10890	2122±460	38;52;60
Cathepsin B[#]	P07858	-6.8	0.316	3351±4321	492±154	9;35;31
Chloride intracellular channel protein 1	O00299					8;27;34
Macrophage capping protein	P40121	-7.0	0.218	855±822	122±54	9;39;27
EH-domain-containing protein 1	Q9H4M9	-7.2	0.196	1364±1196	190±83	14;61;23
Acyl-CoA dehydrogenase[#]	P49748	-7.7	0.068	681±327	88±60	20;70;31
Pyruvate kinase isozymes M1/M2	P14618					13;58;29
Aldo-keto reductase family 1 member C2	P52895	-8.0	0.230	1931±2011	242±67	11;37;20
Leukocyte elastase inhibitor	P30740	-8.4	0.080	1668±894	199±31	14;43;29
Sorting nexin-4[#]	O95219	-9.1	0.218	477±471	53±61	2;52;6
Plastin-3	P13797					4;64;8
Glutathione S-transferase omega 1	Q5TA03	-10.4	0.217	2491±2469	241±109	15;33;38
Calgranulin B	P06702	-11.3	0.220	3869±4009	341±233	6;13;45
Cystatin A	P01040	-11.8	0.012	5618±1282	475±317	2;12;27
Keratintype II cytoskeletal 5	P13647	-13.2	0.124	3676±2607	278±264	29;62;41
Calgranulin A*	P05109	-13.4	0.222	7884±8441	589±416	5;11;41
		-11.5	0.208	5479±5306	474±227	
Galectin-7	P47929	-13.7	0.124	2326±1677	170±125	4;15;33
not identified		-25.0	0.062	821±417	31±7	
Protein-glutamine gamma-glutamyltransferase 2	P21980	-26.8	0.084	1557±934	58±51	15;77;21
not identified		< -30	0.222	942±1089	0	
UDP-glucose 6-dehydrogenase	O60701	< -30	0.125	6603±5255	0	6;56;13
Creatine kinaseubiquitous	P12532	< -30	0.106	3966±2816	0	18;47;31
Glycerol-3-phosphate dehydrogenase	P43304	< -30	0.074	1191±692	0	7;85;10

Protein spots were considered of interest if either the spot intensities showed a significant difference (Student's t-test, FDR-corrected $p \leq 0.05$) between the test group and the controls or if the average spot intensities were at least 4-fold up- or downregulated between the two groups. Furthermore, spots that were uniquely detected in one of the samples, from either control or p53-disrupted cells, were also of interest. *****proteins were detected in corresponding spots of overlapping regions in two gel types;

[#]single spots with two proteins identified, the separate regulation factors could not be distinguished for these proteins; [†, ‡]Proteins with same identity, these were found in spots with different pI/M_r.

A hypergeometric test was performed to assess which Gene Ontology 'molecular function' classes are overrepresented in a statistically significant way. The results are listed in Table 2, for which only gene ontology terms with four or more proteins were selected. As can be seen from this Table, in fact a whole branch of the Gene Ontology 'molecular function' is highlighted, with 'glucose metabolism', 'main pathways of carbohydrate metabolism', and 'energy derivation by organic compound' as the most relevant ones.

Table 2. Overrepresentation of Gene Ontology categories

Gene ontology no.	*Description*	*p-value*	*Proteins SWISS-PROT accession no.*
6006	glucose metabolism	3.12E-06	Q16851 P37837 P04406 P00558 P43304 P06733 O60701 P14618
6092	main pathways of carbohydrate metabolism	3.12E-06	P37837 P04406 P00558 P21912 P43304 P06733 P53396 P14618
15980	energy derivation by oxidation of organic compounds	3.12E-06	P49748 P37837 P04406 P00558 P21912 P43304 P06733 P53396 P14618
44262	cellular carbohydrate metabolism	1.20E-05	Q16851 P37837 P06865 P04406 P00558 P21912 P43304 P06733 O60701 P53396 P14618
19318	hexose metabolism	1.20E-05	Q16851 P37837 P04406 P00558 P43304 P06733 O60701 P14618
5996	monosaccharide metabolism	1.20E-05	Q16851 P37837 P04406 P00558 P43304 P06733 O60701 P14618
6007	glucose catabolism	1.20E-05	P37837 P04406 P00558 P43304 P06733 P14618
5975	carbohydrate metabolism	2.07E-05	Q16851 P37837 P06865 P04406 P00558 P21912 P43304 P06733 Q04760 O60701 P53396 P14618
19320	hexose catabolism	2.14E-05	P37837 P04406 P00558 P43304 P06733 P14618
46365	monosaccharide catabolism	2.14E-05	P37837 P04406 P00558 P43304 P06733 P14618
46164	alcohol catabolism	2.27E-05	P37837 P04406 P00558 P43304 P06733 P14618
44248	cellular catabolism	4.54E-05	P37837 P06865 P04406 P09936 P00558 P21912 P43304 P06733 P53396 P14618 P00505 P49721
16052	carbohydrate catabolism	1.15E-04	P37837 P04406 P00558 P43304 P06733 P14618
44275	cellular carbohydrate catabolism	1.15E-04	P37837 P04406 P00558 P43304 P06733 P14618
9056	catabolism	2.15E-04	P37837 P06865 P04406 P09936 P00558 P21912 P43304 P06733 P53396 P14618 P00505 P49721
6066	alcohol metabolism	4.43E-04	Q16851 P37837 P04406 P00558 P43304 P06733 O60701 P14618
6091	generation of precursor metabolites and energy	7.63E-04	P49748 P37837 P04406 P00558 P21912 P43304 P06733 O60701 P53396 P14618 O76003
44265	cellular macromolecule catabolism	1.04E-03	P37837 P04406 P09936 P00558 P43304 P06733 P14618 P49721
6096	glycolysis	1.15E-03	P04406 P00558 P06733 P14618
9057	macromolecule catabolism	2.51E-03	P37837 P04406 P09936 P00558 P43304 P06733 P14618 P49721

Identified proteins (indicated in Table 1) were analyzed using the software BiNGO[21] to determine the overrepresentation of Gene Ontology 'molecular function' categories of these proteins against the human proteins database. A hypergeometric test was performed and the p-value was adjusted for FDR through Benjamini-Hochberg correction. The chosen significance level was 0.05.

Verification by immunohistochemistry

We chose to use immunohistochemistry for verification of the detected differential expression for proteins that were considered as potential biomarkers, since immunostaining is a method that can be straightforwardly implemented on FFPE material at routine pathology laboratories. Criteria for selection of proteins listed in Table 1 were the extent of regulation and the availability of suitable antibodies. For six of the potential biomarker proteins, i.e. peroxiredoxin-1, manganese-containing superoxide dismutase, lactoylglutathione lyase, FK506-binding protein 5, keratin 5, and keratin 14, the expression pattern seen with 2D-DIGE was confirmed on FFPE specimens of cells transduced with dominant negative mutant p53. Figure 4 depicts the detection of these six proteins by IHC in normal and p53 inactivated cells. Staining of p53 was used as control.

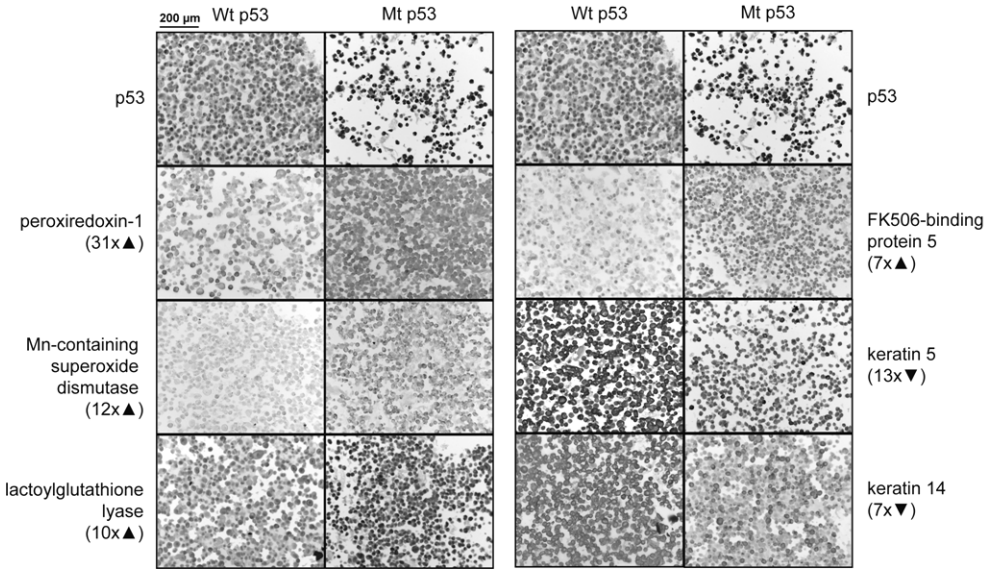

Figure 4. Verification of differential expression in cell model (available in color)

Verification by immunohistochemistry of the differential expression of peroxiredoxin-1, manganese-containing superoxide dismutase, lactoylglutathione lyase, FK506-binding protein 5, keratin 5 and keratin 14 for the p53 inactivated cell model. The regulation of each protein, as determined by 2D-DIGE, is stated between brackets. Positive staining is shown in brown; nuclear counterstaining is in blue. The left-hand panels show wildtype p53 cells (Wt p53) and the right-hand panels mutated p53 (Mt p53). Detection of p53 by IHC is only possible in the squamous cell model with dominant negative p53, for which mutated p53 is overexpressed. Upregulated protein expression is shown by more intense staining and/or a higher percentage of positive cells. Downregulation of protein levels is apparent through fainter staining and/or a reduced percentage of positive cells.

As we were interested in the value of these differentially expressed proteins as potential biomarker, we performed the staining IHC on previously characterized normal and p53 inactivated epithelial tissues from patients. As illustrated in Figure 5, the differential expression of peroxiredoxin-1 could indeed be confirmed in these tissues. For the other protein biomarker candidates, the altered protein expression levels could not be confirmed by IHC in these patient tissues, mostly due to enhanced background staining in the submucosa and inconsistent staining patterns throughout the mucosal layers, which hampered an accurate evaluation of the staining patterns.

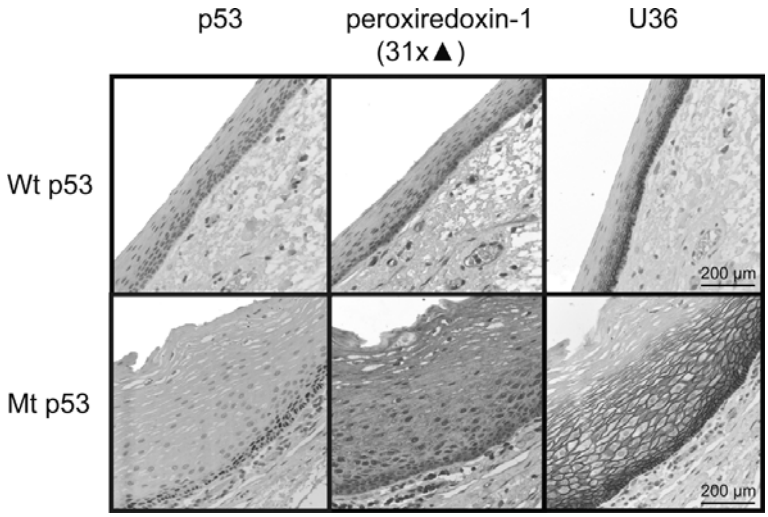

Figure 5. Immunohistochemical staining of peroxiredoxin-1 on patient tissues (available in color) Positive staining is shown in brown; negative counterstaining is in blue. The top panel displays wildtype p53 (Wt p53) and the bottom panel mutant p53 (Mt p53). U36 is a control antibody, directed against CD44v6 that always stains the lower half of the epithelium. Peroxiredoxin-1 clearly demonstrates differential staining of the mucosal cells between normal and mutated p53, in agreement with the over 30-fold increase detected with 2D DIGE. The difference in the appearance between the Wt p53 and Mt p53 tissue sections is mainly due to variation in angle of sectioning.

DISCUSSION

Conditionally immortalized squamous cell model

A novel aspect in this study is the use of a unique and specific conditionally immortalized squamous cell model. With this model, cells can be cultured for a long period and still be forced to return to their primary state at 39ºC. This model enabled us to study the various types of p53 pathway abrogation that also occur in tumors, characterized by inactivation of p53, either through missense mutation or nonsense mutation, or through p53 degradation by the HPV E6 protein. Of these, the missense mutation is most frequently found in tumors. This model permitted detection of 74 protein level changes in response to p53 function disruption, although for most of these proteins no primary relationship could be found with p53 function. Primary effects of p53 function disruption might have been be detected in the nuclear proteome[23], however, due to the limited amount of cells that could be collected, it was not possible to purify the nuclei, in which the primary effects of p53 function disruption may be expected.

Role of p53 in metabolism

A hypergeometric test was performed to assess which of protein classes with differential levels between p53 inactivated cells and control cells were overrepresented. Many of these proteins were overrepresented in our model, in particular enzymes involved in 'glucose metabolism', and 'energy derivation by oxidation of organic compounds' (Table 2). This confirms previous data, since it has been found earlier that p53 has a role in energy metabolism, in particular in regulation of glucose metabolism (reviewed in[24]). Moreover, in virtually all cancers, a metabolic change occurs that results in high rates of glycolysis, known as the Warburg effect. Loss of p53 function in cancer cells is one of the factors that contribute to this Warburg effect. Furthermore, it has also been found that the function of p53 as glycolysis regulator results in modulation of oxidative stress. Although the precise regulation mechanisms are not known, p53 can promote the expression of antioxidant proteins that function to lower reactive oxygen species levels.[25]

Verification of protein expression candidate biomarkers

The most straightforward diagnostic implementation of potential protein biomarkers is to apply these for immunohistochemistry, since this permits detection of precancerous changes in the surgical margins using FFPE specimens. The results were verified in our model for six proteins on both p53 inactivated (dnp53) and wild-type p53 control FFPE cells (Figure 4). Only anti-peroxiredoxin-1 antibody showed the expected result on tissue samples, i.e. that

peroxiredoxin-1 is significantly upregulated (Figure 5; Table 1). The other antibodies displayed enhanced background staining in the submucosa or inconsistent staining patterns throughout the mucosal layers.

Hence, the results suggest that peroxiredoxin-1 is a promising biomarker in tissue samples containing a missense dominant-negative *TP53* mutation. We were not able to confirm peroxiredoxin-1 upregulation in precursor fields in the surgical margins that contain either a *TP53* nonsense mutation or HPV E6. These abrogations of p53 activity occur relatively infrequent, and we did not have these margins in our biobank. As we verified the expression of peroxiredoxin-1 in precursor fields of patient tissues in relation to p53 status, this suggests that the use of our p53 inactivated cell model has potency as predictor for presence of precursor fields. Verification of the other proteins of interest listed in Table 1 awaits development of new suited antibodies.

Peroxiredoxin-1 function related to cancer development

Many of the differentially expressed proteins have also been described in previous studies as candidate biomarkers for HNSCC, like peroxiredoxin-1, pyruvate kinase, manganese-containing superoxide dismutase, transgelin-3 and glycerol-3-phosphate dehydrogenase.[26-29] We detected in our cell model an average of thirty-fold increase in peroxiredoxin-1 expression levels as result of p53 inactivation. Recently, Yaganawa *et al.* also have found that peroxiredoxin-1 expression was associated with local recurrence in tongue squamous cell carcinomas.[30] Furthermore, Yaganawa *et al.* have found that peroxiredoxin-1 may be a tumor marker in oral cancer[31], as significantly higher peroxiredoxin-1 levels were found for T1+T2, N0 and stage I-II case patients. Peroxiredoxin-1, also known as PAG (proliferation-associated gene), is a scavenger of reactive oxygen species, and is known to be involved in the redox regulation of cellular signalling and differentiation.[32] Egler *et al.*[33] have described that peroxiredoxin-1 interacts with c-Myc mediated transformation, suggesting a tumor suppressing role for peroxiredoxin-1. Several other studies have been performed in which raised levels of peroxiredoxin-1 expression in cancerous tissues have been detected, which might be a result of increased oxidative stress or proliferation in tumors.[30, 31, 34, 35] Here we show that this may also be due to the p53 status. Thus peroxiredoxin-1 plays an important role in carcinogenesis and tumor maintenance and may therefore be a valuable biomarker. Future research may focus on study of peroxiredoxin levels under various conditions, such as low oxygen or serum starvation, both in the context of wild type and mutated p53.

In conclusion, we have shown that the combination of a valuable squamous cell model in which p53 function was abrogated, 2D-DIGE and LC-MS/MS enabled us to identify numerous proteins that were differentially expressed after disruption of p53. These proteins

may be potential biomarkers for detection of precancerous fields in the mucosal linings of the upper aerodigestive tract, and should be evaluated in large prospective trials to establish their value to predict development of cancer.

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Chapter 3

Differential proteome analysis of paired normal, premalignant and malignant squamous tissue reveals promising protein biomarkers specifically detecting head and neck precancer

Tieneke B. M. Schaaij-Visser
A. Peggy Graveland
Sharon Gauci
Albert J. R. Heck
Elisabeth Bloemena
C. René Leemans
Ruud H. Brakenhoff
Monique Slijper

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ABSTRACT

The morbidity and 5-year-survival rates of head and neck squamous cell carcinoma (HNSCC) are still disappointing due to the large proportion of patients presenting with advanced stage of disease, and the high frequency of local relapse and second primary cancers that are difficult to treat. This urges the need for early diagnosis of both primary tumors and recurrences. HNSCC arises in genetically altered precancerous fields of which only a small subgroup can be recognized clinically. Often these precancerous fields are microscopically recognized as dysplasia. However, dysplasia grading cannot reliably predict the risk of malignant transformation of precancerous fields for the individual patient. Here, we present a panel of novel potential protein biomarkers to identify precancerous fields with a high risk of progression. The proteomes of genetically characterized normal, precursor and tumor tissues of eight patients were compared by two-dimensional difference in-gel electrophoresis, and protein spots of interest were identified by mass spectrometry. In total we discovered 40 differentially expressed proteins (FDR-corrected $p<0.05$) and the most prominent changes were found between tumor tissue and normal mucosa. Keratin 4, keratin 13, cornulin and small-proline-rich protein 3 showed a significantly decreased expression in tumor tissue, which was confirmed by immunostaining on the same patient tissues. In an independent series, these four proteins could distinguish normal tissues from severely dysplastic tissues ($p<0.01$), which are precancerous fields with a high risk of progression, indicating the potential of these biomarkers to predict malignant transformation. We conclude that these proteins, either solo or in combination, are promising biomarkers for the early diagnosis of progressing precancerous fields.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) develops in the mucosal linings of the upper aero- and digestive tract and is the sixth most common cancer worldwide.[1] Risk factors for developing HNSCC are tobacco and excessive alcohol consumption, and more recently the human papillomavirus has been recognized as an additional risk factor.[2] The five-year-survival rate of HNSCC is still disappointing, mainly because two-thirds of all patients present with advanced stage disease (stage III and IV) and 20-40% of patients develop a local relapse, even when the surgical margins are histologically tumor-free. Clinically these relapses at the primary and adjacent sites are assigned as local recurrence when they develop within three years and at < 2 cm distance of the primary tumor. Relapses not fulfilling these criteria are clinically classified as second primary tumors (SPT). Early diagnosis of both primary tumor and local relapse might greatly improve treatment outcome and survival rates. A major problem hampering diagnostic screening programs that aim at early stage cancer detection, is that these tumors show aggressive growth and progress generally from early stage into advanced stage within weeks, necessitating very short time intervals for a screening program to be effective.

It has been well established that HNSCC is the result of a multistep process characterized by the accumulation of genetic and epigenetic alterations, causing activation of oncogenes and inactivation of tumor suppressor genes.[3] Already in 1996 it was shown that the accumulation of genetic changes is associated with increasing histological abnormalities, and the first genetic progression model for HNSCC was presented.[4] The basis of the model, however, was still histological grading. A few years ago, Braakhuis *et al.*[5] adapted the molecular progression model for HNSCC carcinogenesis. This simplified model indicates that HNSCCs arise in precancerous fields of genetically altered epithelial cells, also called precursor fields. The fields are often characterized by p53 pathway disruption[6-8] and allelic loss at chromosome arms 3p, 9p and 17p. The fields can reach dimensions of up to several centimeters in diameter without any clinical symptoms such as pain or visible changes. A minority of the precursor fields appear as clinically identifiable lesions assigned as leukoplakia and erythroplakia, but the large majority is not macroscopically visible. In a precursor field a tumor can develop that will be diagnosed in the course of the disease. When the tumor is excised, the field is often not completely resected as it is generally large and not visible to the naked eye, and this will cause local relapse in the same or adjacent anatomical region.[8-10] These local relapses, arising in unresected fields, have also been named “second field tumors”.[11] The identification of patients with precancerous fields might therefore improve the early diagnosis of both primary tumors and local relapses. Most

precursor fields can be recognized histologically under the microscope as dysplastic mucosal epithelium and the presence of dysplasia in the margins of surgically removed HNSCC carries a risk of developing local relapse.[12] However, currently only severe dysplasia in the surgical margins is considered an indication for post-operative radiotherapy, but never as single criterion. Even though histopathological grading has limited value to predict the malignant potential in individual cases; it currently is the gold standard for identification and risk assessment of precursor fields.

The availability of protein biomarkers that allow identification of progressing precursor fields would permit investigation of the surgical margins by standardized immunohistochemistry (IHC) methods. Patients with precursor fields at high risk of progression should then receive frequent surveillance during follow-up, which would allow early diagnosis of local relapse. It might even become feasible to identify and grade these fields by analysis of brushed cells, a noninvasive method that might be incorporated in screening programs. However, no such protein biomarkers are available yet. With this study, we aimed to discover a panel of novel protein biomarkers for the identification and risk assessment of precursor fields by comparing normal, precancerous field and tumor tissue at the proteome level. These three tissue types were characterized and classified by genetic analysis and selected from the same patient allowing paired statistical analyses. 2D-DIGE was employed to enable quantitative analysis of multiple small-size patient samples over a large linear dynamic range, and mass spectrometry was used to identify the proteins of which the levels changed significantly from normal to tumor progression. This approach showed to be highly effective, since interesting biomarker candidates could be successfully verified by IHC.

MATERIALS AND METHODS

Selection of tissue material

We analyzed the proteomes of frozen normal, precursor and tumor tissues for changes in protein expression. Patients who were scheduled for surgical treatment of a squamous cell carcinoma in the oral cavity or oropharynx and consented to enrolment were included in the study. Biopsies were taken directly from the surgical specimen and frozen immediately in liquid nitrogen. One biopsy was taken from the tumor and four biopsies from the surrounding mucosal epithelium with a clinically normal appearance. Allelic loss analysis was performed on these biopsies to assess the genetic changes in order to distinguish normal mucosa from precursor field (see below). To increase the chance of detecting focal precursor fields, the biopsies were immunostained for p53 (often overexpressed when mutated) and reviewed for

morphological abnormalities.[9, 10] Guided by p53 immunostaining and morphology, tissues were microdissected for allelic loss analysis and preparation of protein extracts.

To evaluate the discriminative potential of the most promising protein biomarkers, a panel of formalin-fixed paraffin-embedded (FFPE) specimens was selected containing five normal mucosa samples, five severely dysplastic mucosa samples (preneoplastic tissue with a high risk of malignant progression), and five tumor samples.

The study was approved by the Institutional Review Board and in accordance to the Dutch guidelines on the use of human specimens for research. All histological evaluations and dysplasia grading were performed by an experienced pathologist (E.B.) according to the standard criteria of the World Health Organization.[13]

Genetic characterization of tissues

P53 immunostaining

All antibodies were obtained from DAKO (Glostrup, Denmark). Frozen sections of tumor tissue and epithelial margins were fixed for 10 minutes in 4% paraformaldehyde in PBS and pre-incubated for 15 minutes with 2% normal rabbit serum. Primary antibodies against p53 (DO-7) were incubated for 1 hr in the dilution 1:100. The second step was performed with a biotinylated rabbit-anti-mouse antibody (1:500) and in the final step horseradish peroxidase labeled streptavidin-biotin-complex was applied. The staining was developed with diaminobenzidine and H_2O_2 as chromogen. The sections were counterstained with haematoxylin and coverslipped with Kaiser's glycerin. Mouse monoclonal antibody U36 directed against CD44v6 always stains squamous cells independent of genetic changes and was utilized as positive control.[14, 15] Normal mouse IgG was included as negative control.

Allelic loss analysis

Allelic loss analysis decisively distinguished normal tissue from precursor field and was also applied to determine the clonal relationship between precursor field and tumor. Allelic loss analysis was performed as described previously.[16] Briefly, from all selected specimens, 10-20 frozen 10 µm sections were cut, mounted on glass slides and stained with methylene blue/toluidin blue. Areas of interest were manually microdissected under a stereomicroscope. Stroma tissue was collected as source for normal DNA. The first and last two 5 µm sections were stained with haematoxylin and eosin, and immunostained with DO7 for mutated p53 as described above to guide microdissection. Microdissected tissue samples were incubated with proteinase K, and the DNA was isolated and purified by phenol-chloroform extraction followed by ethanol precipitation.

Twelve microsatellite markers were used to assess allelic loss on chromosomes 3p, 9p, 11q and 17p: D3S1766, D3S1029, D3S1293, D9S171, D9S162, D9S157, D11S2002, D11S1369, D11S1883, CHRNB1, TP53 and D17S1866. Ten nanograms of DNA were used to PCR-amplify the microsatellite markers and label the products with fluorescent dye. The ABI 3130 Genetic Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) was employed to separate the PCR products and measure the signal intensities. Allelic loss was assigned if the signal intensity of one of the two alleles decreased with 50% or more as compared to normal control DNA isolated from the stroma.

Proteome analysis by Two-Dimensional Differential Gel Electrophoresis (2D-DIGE)

The proteomes of seven normal, eight precursor and eight tumor tissues were compared in a pairwise manner with 2D-DIGE.[17, 18] For one patient all margins showed genetic changes and a normal sample could not be selected. All equipment and software for 2D-DIGE was obtained from GE Healthcare (Diegem, Belgium), unless stated otherwise.

Protein extraction

For each tissue sample, 30 frozen 10 µm sections were cut, mounted on glass slides and stained with haematoxylin. Relevant epithelial (tumor) parts of the tissues were manually microdissected under a stereomicroscope, and transferred into lysis buffer (30 mM TRIS, pH 8.5, 15 mM NaCl, 1.5 mM $MgCl_2$, 7 M urea, 2 M thiourea, one tablet EDTA-free complete-mini-protease-inhibitor-cocktail/100 ml [Roche, Basel, Switzerland]), snap frozen and stored at -80ºC until further use. Protein lysates were prepared by tip sonication (LABSONIC® M homogenizer, Sartorius Filtratie BV, Nieuwegein, The Netherlands) followed by centrifugation and collection of the soluble fraction. Protein lysates were cleaned from other cellular molecules with the Plus One 2D Clean-up kit (GE Healthcare, Diegem, Belgium), dissolved in labeling buffer (30 mM TRIS, pH 8.8, 7 M urea, 2 M thiourea, 4% CHAPS, one tablet EDTA-free complete-mini-protease-inhibitor-cocktail/100 ml) and the protein content was determined with the Plus One 2D Quant kit (GE Healthcare, Diegem, Belgium).

Protein labeling and 2D-DIGE

Protein lysates were labeled with CyDye DIGE Fluor minimal dyes following the manufacturer's protocol. However, due to the minute amounts of tissue samples, the labeling procedure was scaled down. In brief, 10 µg of protein sample was mixed with 80 pmol of either Cy3 or Cy5, and after 30-minute incubation on ice the labeling reaction was ended by adding 10 nmol of lysine. Equal amounts of all samples were mixed and 10 µg of

this mix was labeled with Cy2 for each internal standard. Samples to be run on the same gel were combined, snap-frozen and stored at -80ºC.

Proteins were separated in two dimensions as described before.[19] In the first dimension, proteins were resolved by IEF using both pH 3-7 (24 cm, non-linear) and pH 6-11 (18 cm, linear) pH-gradients. After equilibration of the pH-strips in buffer (50 mM TRIS, pH 8.8, 6M urea, 2% SDS, 30% glycerol) containing 1% DTT (reduction, 10 minutes) and 2,5% iodoacetamide (alkylation, 10 minutes), the strips were placed on top of a 11% polyacrylamide gel for the second dimension. The gels were scanned on a Typhoon 9400 Imager and the gel images were cropped with ImageQuant TL software (version 2005).

DeCyder analysis of 2D gel images

The spot co-detection, quantification by normalization and ratio calculation for the gel images from one single gel was performed in the Differential In-Gel Analysis (DIA) module of the DeCyder 2D software (version 6.5). For spot detection the V6 spot detection algorithm was used with the estimated number of spots set to 10,000 and the exclude filter set to >30,000 for volume. In the Biological Variation Analysis (BVA) module of DeCyder, all the gel images were processed and matched. To select protein spots of which the intensities were significantly different between the normal, precancerous and tumor tissues, a paired one-way ANOVA was performed on the whole sample group. Paired Student's t-tests were applied to detect differential protein spot intensities between either normal and precancerous tissue, normal and tumor tissue or precancerous and tumor tissue, and to calculate average ratios between each of these two tissue types. P-values were corrected for the false discovery rate (FDR). Proteins were considered differentially expressed when the FDR-corrected p-values of the ANOVA analysis were <0.05. Hierarchical clustering of the samples was done in the Extended Data Analysis (EDA) module of DeCyder.

Protein identification by LC-MS/MS

Spots from differentially expressed proteins were picked from the analytical gels with the Ettan Spot Picker (GE Healthcare, Diegem, Belgium) according to the manufacturer's protocol. The gel plugs were washed in milliQ water followed by tryptic in-gel digestion as described.[20] Peptides were extracted with 5% formic acid to a total volume of 20 µl.

Ten µl of peptide sample was analysed on either a nanoLC-LTQ-Orbitrap-MS or a nanoLC-LTQ-FT(ICR)-MS (both Thermo Electron, San Jose, CA) as described before.[21, 22] All MS/MS files were merged into a single file that was used as input for the MASCOT search engine (version 2.2.04, Matrix Science, London, U.K.). Spectra were searched against the IPI_Human_3.36 database that contained 69,012 *Homo sapiens* protein sequences. Further

search settings were: trypsin digestion with maximal one missed cleavage, carbamidomethyl (C) and oxidation (M) as fixed and variable modification, respectively, peptide tolerance of 5 ppm with 1+, 2+ and 3+ peptide charges and MS/MS tolerance of 0.9 Da. A Mascot cut-off score of 30 was set for peptides and a minimal of two peptides were required for identification of a protein. All protein spots were excised and analyzed by MS at least three times.

Evaluation of discriminative potential of promising biomarker candidates

The monoclonal mouse antibodies directed against keratin 4, keratin 13 and small proline-rich protein 3 were from Abcam (Cambridge, UK). The goat antibody against cornulin was from R&D Systems (Abingdon, UK). Antibodies were used in dilution 1:100 for keratin 4, 1:100 for keratin 13, 1:10 for small proline-rich protein 3 and 1:200 for cornulin. All other antibodies were from DAKO (Glostrup, Denmark).

Paraffin sections were deparaffinized, rehydrated, subjected to antigen retrieval by microwave boiling for 10 minutes in 10 mM TRIS pH 9.0, 1 mM EDTA, and pre-incubated for 15 minutes with 2% normal rabbit serum. Primary antibodies were incubated for 1 hr. The second step was performed with a biotinylated rabbit-anti-mouse or rabbit-anti-goat antibody (1:500) and in the final step horseradish peroxidase labeled streptavidin-biotin-complex was applied. The staining was developed with diaminobenzidine and H_2O_2 as chromogen. The sections were counterstained with haematoxylin and coverslipped with Kaiser's glycerin. Positive (normal mucosa) and negative (tumor) controls were added with each immunohistochemical assay to ensure reproducibility of the antibodies. Mouse monoclonal antibody U36 and normal mouse IgG were included as positive and negative controls, respectively.

Quantitative scoring of the immunostained sections was carried out by at least two independent observers. Staining intensity was divided into no staining (=0), moderate staining intensity (=1) and strong staining intensity (=2). Staining results were calculated by multiplying the staining intensity with the estimated percentage of stained cells, resulting in a range from 0 to 200. Significant differences in staining intensity were calculated by Student's t-test.

RESULTS

Patient and tissue selection

Mucosal tissues surrounding tumors frequently show genetic changes indicative of the presence of a precancerous field. To select genetically normal mucosa, a precancerous field and tumor of the same patient, we applied a detailed allelic loss analysis. Genetic characterization by allelic loss analysis ensured the selection of normal and precursor epithelium, and confirmed the clonal relation between precursor and tumor tissue. Figure 1 shows an overview of the sample selection process and gives a typical example of allelic loss profiles for the normal, precancerous and tumor tissue from one patient.

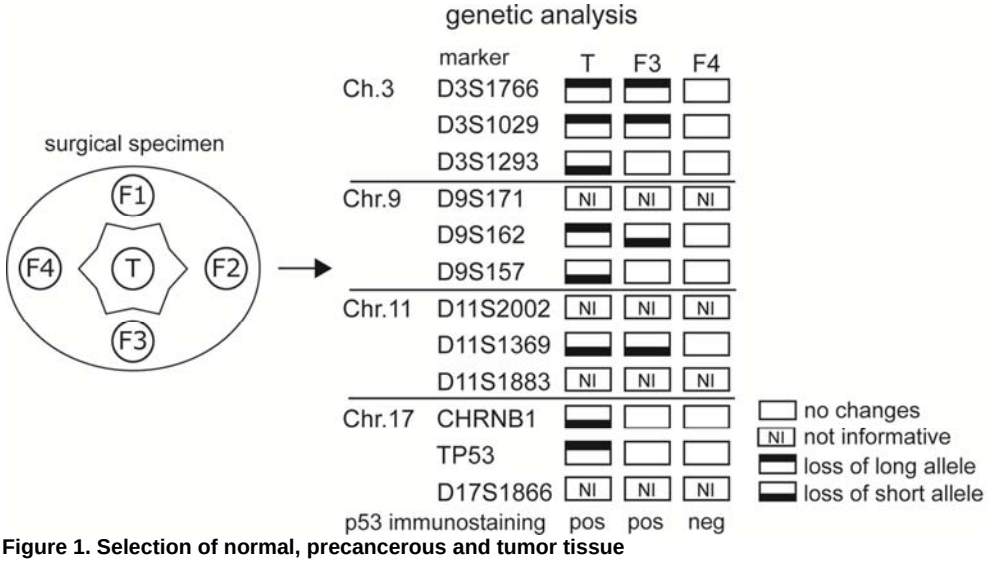

Figure 1. Selection of normal, precancerous and tumor tissue

From the surgical specimen, one biopsy was taken from the tumor and four from the surrounding mucosa. All samples were evaluated by histopathology and immunostaining. Patients with dysplasia (mild/moderate) and/or positive p53 mutation staining in one or more of the biopsies were selected for genetic analysis. Microsatellite markers on chromosome arms 3p, 9p, 11q and 17p were used to assess genetic changes. Allelic loss of these chromosomes is known to be an early event in squamous cell carcinogenesis. A typical example of these genetic patterns is depicted. In this case, the tumor contained allelic loss of all informative markers and F3 showed a similar genetic pattern, however, not all markers are involved. F3 was considered a precancerous field and F4 normal mucosal tissue.

From a total of seventeen patients, tumor sections and sections of two to four mucosal biopsies were evaluated by immunostaining for p53 overexpression. Eight patients were selected for allelic loss analysis, based on positive p53 mutation staining in one or more of mucosal biopsies. For each of the selected patients, allelic loss was detected in the tumor

sample and in at least one of the mucosal biopsies. For every patient one mucosal biopsy with (precursor tissue) and one without (normal tissue) allelic loss were selected. For one patient, however, allelic loss was detected in all mucosal biopsies, and therefore no normal tissue was available. Table 1 lists the eight patients used for this study, their characteristics and the selected tissues.

Table 1. Characteristics of selected patients and tissues

Patient	*Gender*	*Age*	*Site*	*Subsite*	*T-stage*	*Precursor tissue*	*Normal tissue*
						p53 staining; allelic loss	
1	m	46	oral cavity	mobile tongue	T2N2BM0	No;9p	Yes*
2	m	48	oral cavity	mobile tongue	T2N0M0	Yes;3p,9p	Yes*
3	m	67	oral cavity	mobile tongue	T1N0M0	No;9p	Yes*
4	m	43	hypopharynx	piriform sinus	T4N2BM0	Yes;3p,9p,11q	No
5	m	78	oral cavity	mucosa lower jaw	T4N1M0	No;3p	Yes*
6	f	64	oral cavity	retromolar trigone	T2N2BM0	Yes;9p,11q	Yes*
7	m	55	larynx	glottic larynx	T4N0M0	Yes;11q,17p	Yes*
8	m	77	oropharynx	retromolar trigone	T4N0M0	Yes;3p,9p,11q,17p	Yes*

* Mucosal tissues were classified as normal when p53 immunostaining was negative and no allelic loss was detected

2D-DIGE analysis and identification of differential proteins

The Cy2 gel images of the internal standard in Figure 2 indicate the high number of protein spots and the high quality protein separation of 2D-DIGE, considering the low amounts of protein that were used (10 µg). In total approximately 1,000 protein spots could be matched between all the tissue samples (pH-ranges 3-7 and 6-11 combined). Seventy protein spots showed statistically significant differences in intensity in either normal, precancerous or tumor tissue, with some of the ratios between normal and tumor tissue exceeding 10-fold (50 spots in pH-range 3-7, 24 spots in pH-range 6-11, 4 spots were in the overlapping pH-range of both the 3-7 and the 6-11 gels). As can be concluded from Table 2, the largest changes in expression level were found between normal and tumor samples. In total 40 unique proteins could be identified in these 70 differential protein spots. Thirteen proteins were present in multiple spots (with comparable differential expression levels) and in four spots no proteins could be identified by mass spectrometry, most likely due to the minute amount of protein present.

Identified proteins are involved in various cellular processes, like epidermis development and epithelial differentiation, protein synthesis, protein folding and stress response (ER-chaperone proteins), and cellular organization and structuring (indicated in Table 2). A prominent functional group of proteins is related to cellular differentiation processes, e.g. the keratin couple 4/13, cornulin and small proline-rich protein 3. For all these proteins the levels

decreased significantly in tumor tissue, as shown in Figure 2C, and reflected by low FDR-corrected p-values for the ANOVA and Student's T-test (Table 2).

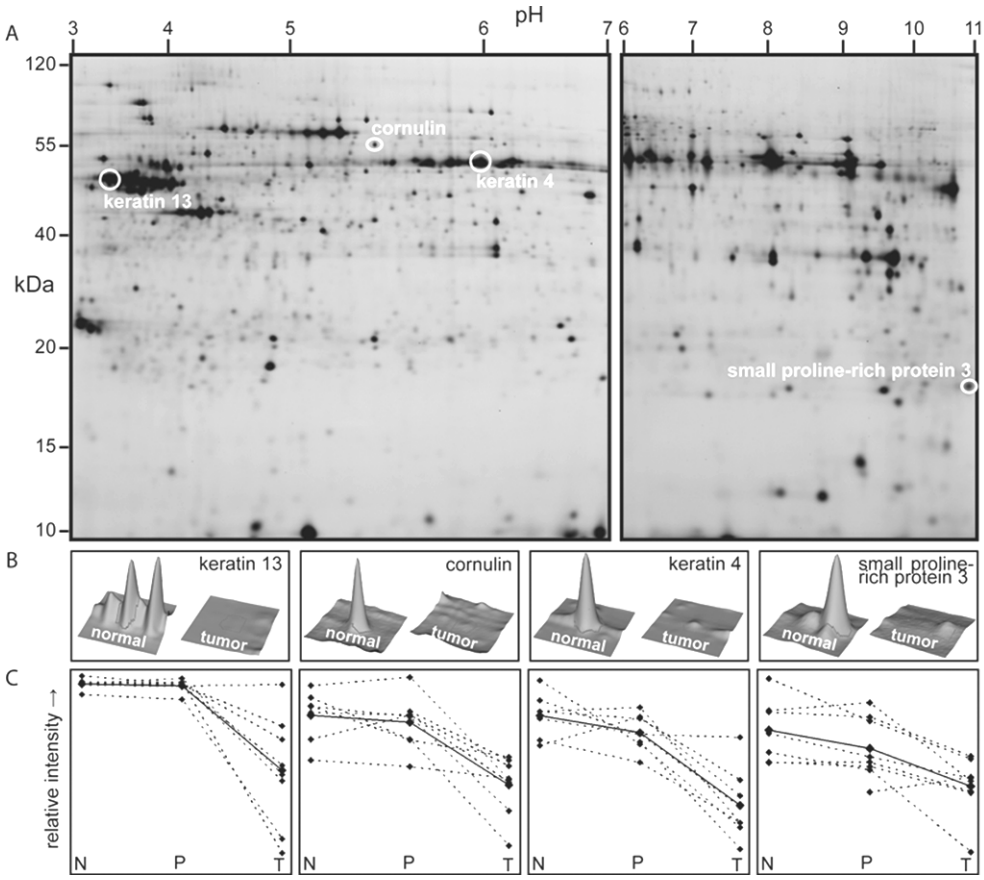

Figure 2. Differential protein spot intensities as identified by 2D-DIGE analysis

The proteomes of normal, precancerous and tumor tissue of 8 patients were compared by 2D-DIGE (of one patient normal tissue was not available as all margins showed a precursor field). A: Typical 2D-DIGE internal standard gel images for pH-range 3 to 7 and 6 to 11. Overall approximately 1,000 proteins could be matched over all the samples. Four differentially expressed proteins of interest for validation are indicated in the images. B: 3D spot images of the differential proteins in gel images of the normal and the tumor tissue samples from one single patient as example. C: Relative protein expression levels for the differential proteins (data are normalized by the DeCyder software). Dashed lines indicate the values of the tissue samples derived from single patients; continuous lines indicated the average values. N = normal tissue, P = precursor tissue and T = tumor tissue.

Table 2. List of differential proteins

Protein name	*Accession number*	*pH range*	*ANOVA p-value*	*N/P (r,p-value)*	*P/T (r, p-value)*	*N/T (r, p-value)*	*kDa*	*pI*
Epidermis development / epithelial differentiation								
Cornulin	Q9UBG3	3-7	0.004	-1.26, 0.68	-4.23, 0.017	-5.32, 0.016	54	5.8
Keratin, type I cytoskeletal 13#	P13646	3-7	0.002	-1.15, 0.76	-5.08, 0.017	-8.99, 0.010	50	4.9
Keratin, type II cytoskeletal 4#	P19013	3-7	0.008	1.07, 0.76	-4.68, 0.018	-9.54, 0.011	64	8.5
Small proline-rich protein 3#	Q9UBC9	6-11	0.006	-1.14, 0.81	-3.84, 0.078	-3.94, 0.033	18	8.9
Protein synthesis								
Elongation factor 1-alpha 1	P68104	6-11[1]	0.018	1.11, 0.78	1.52, 0.173	1.63, 0.0356	50	9.1
Elongation factor 1-delta	P29692	3-7	0.024	1.41, 0.58	1.20, 0.180	1.63, 0.013	31	4.9
Eukaryotic translation initiation factor 3 subunit I	Q13347	3-7	0.008	1.43, 0.63	1.88, 0.036	2.50, 0.010	37	5.4
Eukaryotic initiation factor 4A-III	P38919	3-7[2]	0.009	1.11, 0.86	1.76, 0.015	1.88, 0.017	47	6.3
Protein folding and stress response								
DnaJ homolog subfamily B member 1	P25685	6-11	0.014	-1.04, 0.88	-1.50, 0.134	-1.59, 0.043	38	8.7
DnaJ homolog subfamily B member 11	Q9UBS4	3-7	0.000	1.19, 0.60	1.84, 0.015	2.28, 0.008	41	5.8
Endoplasmin	P14625	3-7	0.017	1.48, 0.68	3.18, 0.031	4.58, 0.025	92	4.8
Endoplasmic reticulum protein ERp29	P30040	3-7	0.005	1.57, 0.44	-1.05, 0.250	1.51, 0.015	29	6.8
Peptidyl-prolyl cis-trans isomerase B	P23284	6-11	0.002	1.1, 0.88	1.74, 0.052	1.90, 0.021	24	9.3
Protein disulfide-isomerase A4	P13667	3-7	0.005	1.21, 0.76	2.81, 0.026	3.08, 0.010	73	5.0
Serpin H1 precursor#	P50454	6-11	0.000	1.38, 0.56	5.25, 0.057	6.54, 0.007	46	8.8
Stress-induced-phosphoprotein 1	P31948	3-7	0.006	1.89, 0.58	1.82, 0.047	2.84, 0.010	63	6.4
Thioredoxin domain-containing protein 5	Q8NBS9	3-7	0.008	1.35, 0.64	2.17, 0.044	2.97, 0.015	48	5.6
Cellular organization and structuring								
Annexin A1#	P04083	3-7	0.001	-1.43, 0.59	-2.61, 0.024	-5.08, 0.009	39	6.6
Annexin A5#	P08758	3-7	0.002	1.55, 0.58	2.37, 0.017	3.45, 0.010	36	4.9
Lamin-A/C	P02545	3-7	0.002	1.41, 0.58	2.02, 0.014	2.88, 0.011	74	6.6
Lamin-B1	P20700	3-7	0.015	1.6, 0.58	1.62, 0.058	2.45, 0.022	66	5.1
Myosin light chain 1#	P05976	3-7	0.002	-1.26, 0.59	-4.61, 0.016	-5.23, 0.014	21	5.0
Septin-7#	Q16181	6-11	0.007	1.16, 0.78	1.68, 0.109	1.91, 0.030	51	8.7
Vimentin	P08670	3-7	0.002	1.65, 0.59	2.11, 0.017	3.07, 0.008	54	5.1
Other								
Adenylosuccinate synthetase isozyme 2	P30520	3-7[2]	0.009	1.11, 0.86	1.76, 0.015	1.88, 0.017	50	6.1
Alcohol dehydrogenase class 4 mu/sigma chain	P40394	6-11	0.035	1.04, 0.88	-1.86, 0.135	-2.18, 0.037	41	8.1
Bifunctional purine biosynthesis protein PURH	P31939	3-7	0.005	1.37, 0.59	1.78, 0.024	2.29, 0.010	65	6.3
Glutathione S-transferase kappa 1	Q9Y2Q3	6-11	0.009	1.07, 0.97	-1.68, 0.057	-1.63, 0.033	25	8.5
Interleukin-1 receptor antagonist protein	P18510	3-7	0.004	-1.29, 0.66	-2.33, 0.043	-4.56, 0.008	20	5.8
Isocitrate dehydrogenase [NADP]#	P48735	6-11	0.010	1.05, 0.89	1.62, 0.063	1.73, 0.051	51	8.9
Lactoylglutathione lyase#	Q04760,	3-7	0.008	-1.26, 0.65	-2.22, 0.021	-2.71, 0.020	21	5.1
L-lactate dehydrogenase A chain#	P00338	6-11	0.009	1.05, 0.88	1.72, 0.096	1.80, 0.038	37	8.4
Phosphatidylinositol transfer protein beta	P48739	3-7	0.034	1.56, 0.58	-1.19, 0.083	1.23, 0.130	32	6.4
Plastin-2	P13796	3-7	0.017	1.97, 0.58	1.77, 0.066	3.24, 0.024	70	5.2
Sulfide:quinone oxidoreductase	Q9Y6N5	6-11[1]	0.018	1.11, 0.78	1.52, 0.173	1.63, 0.036	50	9.1
Superoxide dismutase [Mn]#	P04179	3-7	0.003	1.83, 0.37	2.17, 0.038	4.11, 0.013	25	8.4
Transforming growth factor-beta-induced	Q15582	6-11	0.011	1.29, 0.84	2.84, 0.096	3.68, 0.043	75	7.6
Tropomyosin alpha-4 chain (TPM3)	P67936	3-7	0.004	1.55, 0.58	2.07, 0.043	3.11, 0.010	29	4.7
Tryptophanyl-tRNA synthetase	P23381	3-7	0.017	1.94, 0.42	2.86, 0.060	4.65, 0.024	53	5.8
UMP-CMP kinase#	P30085	3-7	0.008	-1.2, 0.68	-2.54, 0.025	-3.22, 0.017	26	8.1
Not identified		3-7	0.002	-1.53, 0.62	-3.94, 0.015	-6.41, 0.010		
Not identified		3-7	0.002	1.24, 0.78	2.44, 0.013	2.70, 0.011		
Not identified		3-7	0.009	1.01, 0.99	1.65, 0.018	1.64, 0.022		
Not identified		6-11	0.080	27.84, 0.56	-1.26, 0.223	71.15, 0.170		

[1,2] Single spots with two proteins identified, the separate ratios could not be distinguished for these proteins; # Proteins identified in multiple spots, values derived from that spot with the median ANOVA p-value were chosen; N = normal, P = precancerous, T = tumor, r = ratio (a positive value indicates an increase and a negative value indicates a decrease), p-values are FDR corrected.

Figure 3 presents the results of the hierarchical clustering of the tissue samples based on the differentially expressed proteins. Two main clusters can be distinguished. Cluster 1 contains all normal and precancerous fields including one tumor. Cluster 2 contains seven tumor samples. This indicates that normal and precursor mucosal tissue are hard to distinguish at the protein level, while the tumors form a distinct group. The tumor from patient 2 did not cluster with the other tumors. Histopathology revealed that this particular tumor was well-differentiated with presence of a large number of keratin pearls (clusters of keratinized epithelial cells), which might explain its clustering to the normal and precursor tissues.

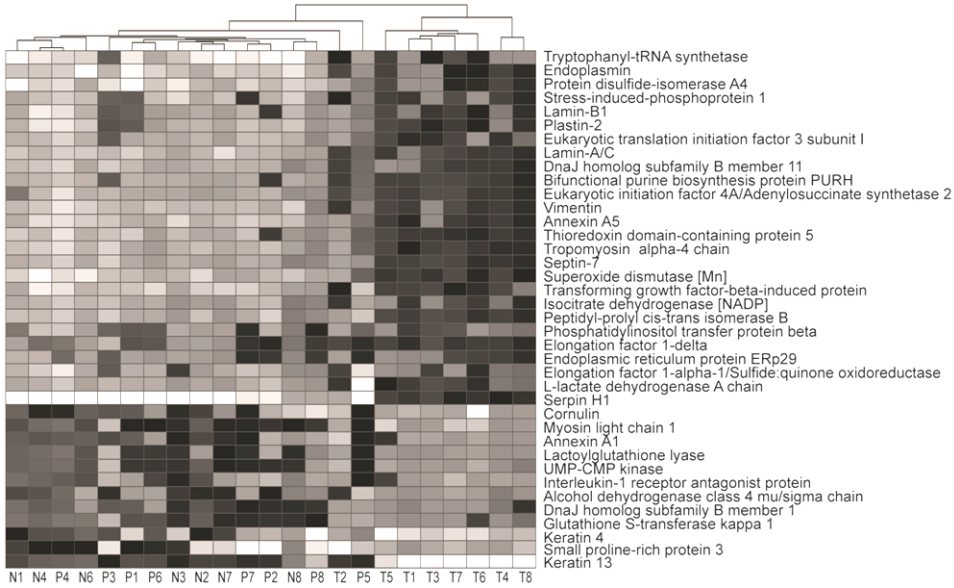

Figure 3. Heatmap showing hierarchical clustering of patient tissue samples based on the differential protein spots (available in color)

Green and red colors indicate relatively decreased and increased expression, respectively. Tissue samples are indicated by type (N=normal, P=precancerous, T=tumor) and patient number (1 to 8). All tumors except for one (T2) classify into one cluster, while normal and precancerous fields are intermingled. This classification indicates that the largest differences are found between tumor and mucosal samples independent of whether it was normal or precancerous field tissue.

Confirmation of differential expression

We selected the proteins with highest tumor to normal ratios and most significant FDR-corrected p-values for further analysis and were able to obtain antibodies against 12 of these proteins. To achieve optimal staining without background signals on FFPE tissue sections, dilutions of all primary antibodies were evaluated using varying incubation times and three antigen retrieval protocols. Four antibodies, directed against keratin 4, keratin 13,

cornulin and small proline-rich protein 3, showed specific staining patterns on sections of FFPE specimens of normal mucosa and tumours, and these four were further explored. Quantification with 2D-DIGE revealed that the expression of keratin 4 and keratin 13 is approximately 9-fold decreased in tumors compared to normal tissue (Table 2). Further, the proteins cornulin and small proline-rich protein 3 were found to decrease approximately 5 and 4-fold in tumors, respectively. Detection of keratin 4, keratin 13, cornulin and small proline-rich protein 3 by immunohistochemistry in the selected series of patient tissues demonstrated a very pronounced expression in the upper layers of the normal mucosa and precursor mucosa and absence of expression in tumor tissue (data not shown), which is in accordance with the results from the 2D-DIGE data.

Discriminative potential of differential proteins

To explore the potential of keratin 4, keratin 13, cornulin and small proline-rich protein 3 expression for the detection of progressing precancerous fields, an independent series of surgical margins were selected from the pathology archive that contained mucosal epithelium graded as severe dysplasia. Severe dysplasias are precursor fields that show multiple genetic changes[23] and have a relatively high risk of malignant progression. These margins were immmunostained with the biomarker panel, and compared to normal and tumor tissues. Examples of the staining images and the semi-quantitative scoring results are depicted in Figure 4. The semi-quantitative expression of all four potential biomarkers differed significantly between normal epithelium and tumor tissue and between normal and severely dysplastic epithelium ($p<0.01$). This validation experiment in an independent series of samples showed the potential of these four proteins to individually distinguish between normal mucosa and mucosa considered to be at high risk of developing cancer.

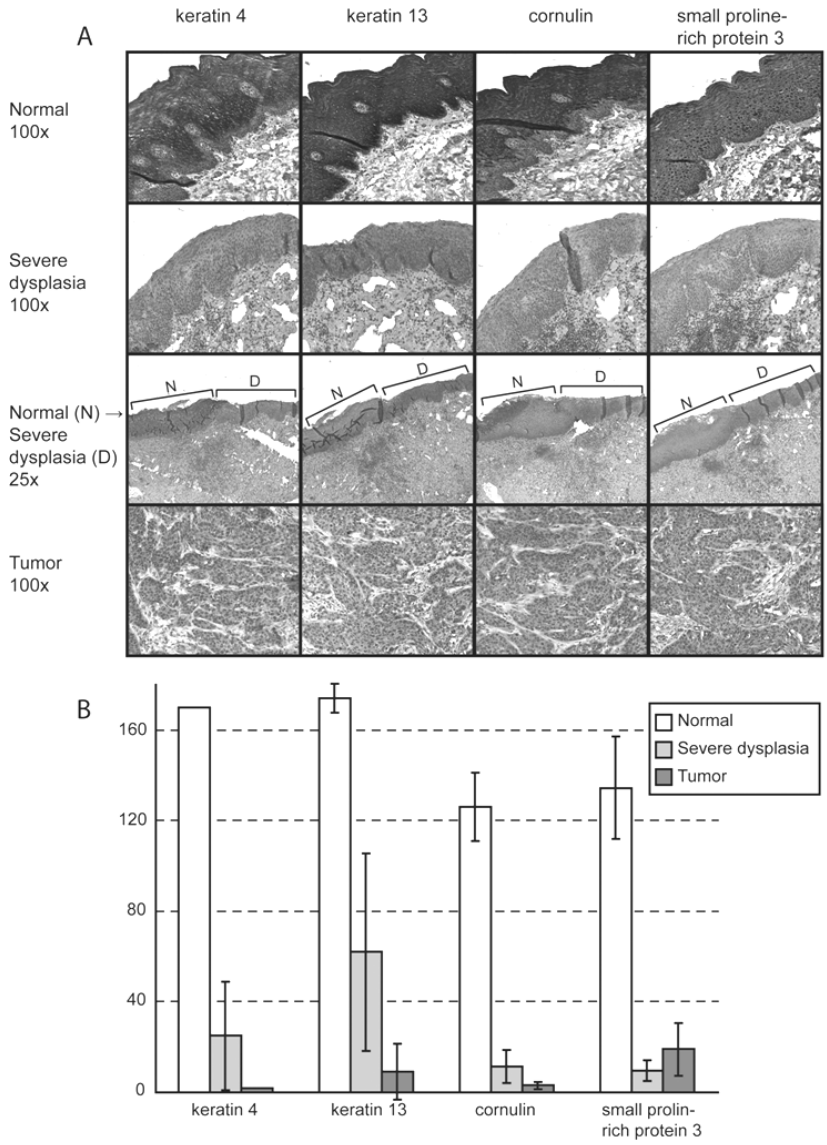

Figure 4. Validation of differential expression of keratin 4, keratin 13, cornulin and small proline-rich protein 3 (available in color)

A: Typical examples of keratin 4, keratin 13, cornulin and small proline-rich protein 3 staining on normal epithelium, severe dysplasia, and tumor tissue; B: Averaged semi-quantitative scoring results of IHC staining of keratin 4, keratin 13, cornulin and small proline-rich protein 3 on an independent set of 5 normal epithelia, 5 severe dysplasias and 5 tumors. Staining intensity was divided into no staining (=0), moderate staining intensity (=1) and strong staining intensity (=2). Staining results were calculated by multiplying the staining intensity with the estimated percentage of stained cells, resulting in a range from 0 to 200. The expression of all four protein differs significantly between both normal and tumor, and between normal and severe dysplasias ($p<0.01$).

DISCUSSION

Proteomics approaches applied on clinical material are hindered by the relatively small amount of protein that is available from tissue samples, and by the need to compare multiple samples in one group (e.g. normal, tumor) to account for genetic variation among individuals.[24, 25] We found earlier that the 2D-DIGE technology has proven to be a valuable platform to solve these issues[17, 18, 24], although it restricts the analysis to the approximately 1,000 highest abundant proteins. With only 10 µg of protein per sample, high quality 2D gel images were obtained, and 24 different patient tissues could be compared in a paired analysis. Four differential spots were quantified in gels from both pH-gradient 3 to 7 and 6 to 11, with similar results (see Table 3), demonstrating the reliability of the findings and the small technical variation.

Table 3. Quantification of similar protein spots, detected within two different pH-ranges

Spot	*Protein name*	*pH-range*	*ANOVA p-value*	*N/P ratio#*	*N/P p-value*	*P/T Ratio#*	*P/T p-value*	*N/T Ratio#*	*N/T p-value*
A	Annexin A1	pH3-7	0.00031	-1.45	0.59	-3.11	0.021	-7.01	0.0083
		pH6-11	0.00132	-1.33	0.775	-3.55	0.057	-7.88	0.0136
B	Annexin A1	pH3-7	0.0012	-1.43	0.59	-2.61	0.024	-5.08	0.0091
		pH6-11	0.00100	-1.56	0.709	-3.68	0.057	-10.07	7.14E-03
C	Keratin, type II cytoskeletal 4	pH3-7	0.0075	1.07	0.76	-4.68	0.018	-9.54	0.011
		pH6-11	0.00396	1.14	0.879	-4.93	0.057	-7.96	0.0158
D	Superoxide dismutase [Mn]	pH3-7	0.003	1.83	0.37	2.17	0.038	4.11	0.013
		pH6-11	0.03490	1.08	0.931	1.94	0.173	1.93	0.0442

a positive value indicates an increase and a negative value indicates a decrease

Recently, a number of proteomics studies were reported on HNSCC cell lines, tissues, saliva and secretomes in order to find biomarkers for head and neck cancer.[26-34] In most of these studies normal samples were compared to tumor samples, or precancerous tissue was defined by abnormal histology. To our knowledge, our study is the first to use detailed genetic analyses to ascertain the selection of strictly normal and precancerous tissues and confirm the clonal relationship between precursor and tumor tissue prior to the proteome analysis. Ralhan *et al.* presented proteome data on oral premalignant lesions as characterized by abnormal histopathology. Their list of differential proteins contained some of the proteins we identified as well, however, the majority of proteins does not overlap.[35] Moreover, they used different approaches for sample preparation, fractionation and for quantification, which is known to cause complementary results. Also, Roesch-Ely *et al.* employed SELDI-TOF-MS to detect differences in protein expression between biopsies from head and neck tumor tissue, normal mucosa, tumor-adjacent mucosa and tumor-distant mucosa.[32] Clearly, there are no overlapping proteins between their dataset and our list of

differentially expressed proteins, which probably results from the two completely different proteomics approaches. SELDI analyses merely aim at finding specific protein profiles, and in general only proteins with low molecular weight can be identified.

To increase the statistical power, we analyzed paired samples from eight different patients. The p-values between our samples reached levels smaller than 0.001, even when corrected for FDR, indicating the strength of the chosen approach. FDR corrections are required as the number of samples is relatively low in comparison to the number of variables (proteins) measured.

Keratin 4 and 13 are a well-known keratin couple present in the suprabasal layer of mucosal stratified squamous epithelial cells.[36] As for all keratin couples, the proteins form a heterotetramer containing two neutral type II keratins (keratin 4) and two acidic type I keratins (keratin 13). Keratin 4 and 13 are best known for their role in the hereditary disorder white sponge nevus, which can be caused by mutations in either one of these keratins.[37] This disease causes benign white lesions in the oral cavity that are easily mistaken for leukoplakias, which are known precancerous changes. Hence, this apparent change in keratin expression might well explain that some precancerous fields present as visible white lesions. Since these proteins are involved in epithelial cell differentiation, they are often found to be downregulated in epithelial tumors, and more specifically, head and neck tumors.[28, 38-40] For example, Ohkura *et al.* described a significant lower mRNA and protein expression of both keratin 4 and 13 in HNSCC.[40]

Both cornulin and small proline-rich protein 3 are present in differentiating keratinocytes. They belong to the epidermal differentiation complex (EDC) locus at chromosome 1q21 and are part of the cornified envelope.[41] Cornulin has recently been identified as one the “fused genes” in the EDC. The genes in this complex are classified in three groups. One contains proteins with short tandem repeated peptide motifs that are cross-linked in the formation of the cornified envelope, an important protective barrier of the mucosa and skin. The small proline-rich proteins belong to this group. The second group of proteins encoded in the EDC locus contains proteins with EF domains that bind calcium. The third group, to which cornulin belongs, contains the fused genes that both have EF domains and short tandem repeats. Cornulin is described to be involved in induction of apoptosis and response to stress and often found to be downregulated in head and neck cancer.[41-44] Small proline-rich protein 3 is also reported to be involved in apoptosis and to be downregulated in tumors.[28, 45] So far, apart from differential protein or mRNA expression, no reports on the prognostic value of either of these two proteins are available yet.

We noted that small differences were found between normal tissue and precancerous tissue, both by 2D-DIGE and by IHC of the differential proteins. The most logical explanation

for this observation is that we selected precancerous fields that were early in the carcinogenic process. We used genetic markers (allelic losses of chromosome arms 3p, 9p, 11q and 17p) that are known to detect early and late changes to ensure selection of truly normal, precancerous and tumor tissues. Moreover in none of the surgical margins severe dysplasia was observed, most likely as severe dysplasia is relatively rare.[23] Therefore, the precancerous tissue samples were well-defined early stages of progression, which explains why only small differences were detected as compared to normal mucosa.

We decided to subsequently analyze the expression of keratin 4, keratin 13, cornulin and small proline-rich protein 3 in selected severe dysplasias, as these carry a high risk of malignant progression. In this independent series immunohistochemical staining of the four proteins revealed a clear distinction between normal and severe dysplastic tissue, supporting the potential of these markers to identify precursor fields with a high risk of progression. Interestingly, Yanagawa *et al.* described an association between absence of keratin 13 immunostaining in surgical margins and local recurrence, strongly supporting the prognostic value of keratin 13.[46] However, large-scale studies of the predictive value of these protein markers are not available yet. Prognostic studies to predict either malignant transformation of leukoplakia lesions or local relapse of unresected precursor fields in surgical margins of treated head and neck cancer patients should be carried out to proof that these markers are able to identify progressing precursor lesions by a simple immunostaining.

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Chapter 4

Evaluation of cornulin, keratin 4, keratin 13 expression and grade of dysplasia for predicting malignant progression of oral leukoplakia

Tieneke B. M. Schaaij-Visser
Jantine F. Bremmer
Boudewijn J. M. Braakhuis
Albert J. R. Heck
Monique Slijper
Isaäc van der Waal
Ruud H. Brakenhoff

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ABSTRACT

Oral leukoplakia is defined as a white patch in the oral cavity that cannot be diagnosed as any other known disorder. These lesions carry an increased risk of malignant progression, and approximately 2-3% per year do progress to cancer. At present biopsies are histopathologically graded for dysplasia to assess the risk of progression, but this grading is somewhat subjective and of limited use for the individual patient.

In a previous study we discovered by a comprehensive proteomics approach that compared to normal mucosa, protein expression of cornulin, keratin 4 and keratin 13 is decreased in tumors and severe dysplasia, preneoplastic tissue with a high risk of malignant progression. Here, we studied whether loss of expression of these proteins can predict malignant transformation of oral leukoplakia. Biopsies of 12 progressing and 36 non-progressing leukoplakia lesions were analyzed for cornulin, keratin 4 and keratin 13 expression by immunohistochemistry, and graded for dysplasia. Kaplan-Meier analysis showed that loss of expression of neither cornulin ($p=0.075$), keratin 4 ($p=0.789$) nor keratin 13 ($p=0.732$) was significantly associated with the risk of malignant transformation of leukoplakia lesions. However, decreased expression of these proteins was significantly associated with the presence of hyperkeratosis. Only dysplasia grading correlated significantly with malignant progression of leukoplakia ($p=0.024$).

Despite the promising outlook that decreased cornulin, keratin 4 and keratin 13 expression in the oral mucosa is associated with a premalignant state, these markers do not predict malignant transformation of leukoplakia lesions. Most likely explanation is that the aberrant differentiation state of hyperkeratotic leukoplakia lesions already causes a decreased expression, obscuring the putative association with malignant transformation. Our results support the significance of dysplasia grading for the prediction of malignant transformation.

INTRODUCTION

In 2007, oral leukoplakia was defined by the WHO as 'white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk of cancer'.[1] In other words, leukoplakia is a white patch in the oral cavity that cannot be classified as any other known disorder, and has an increased risk to develop into cancer. Leukoplakia has a prevalence of an estimated one to eight percent depending on age as well as gender, and most lesions arise in the mandibular or buccal mucosa.[2] The reported malignant transformation rates vary considerably between studies, depending both on the clinical definitions used and the follow-up time.[3] Previously, it has been reported that 2-3% of all leukoplakias progress to cancer every year. After 16 years of follow-up this still accumulates to 50%.[4]

The clinical management of leukoplakia currently consists of initial biopsy followed by either excision or CO_2 laser treatment, or watchful waiting. Both treated and untreated patients will receive frequent (every three months) or less frequent (every 6 months) follow-up surveillance, depending on the presence of epithelial dysplasia in the biopsy.[5,6] Histological grading of epithelial dysplasia is still the gold standard to predict malignant transformation, but it is somewhat subjective and has limited value in individual cases. The used interventions have limited effects both on the recurrence rate of the lesions as well as on the risk of malignant transformation.[7]

Cancer in general, and also oral cancer is caused by the accumulation of genetic alterations such as chromosomal losses and gains[8-10], and these genetic changes have been used to predict the risk of malignant transformation of oral leukoplakias. Several studies have shown that the presence of allelic loss can be used to discriminate leukoplakias with a low risk from those with a high risk of malignant transformation.[11-13] Unfortunately, genetic analysis is laborious and time-consuming, and cannot be readily implemented in diagnostic laboratories. A relatively simple immunostaining approach would be ideal to complement or replace histological grading of epithelial dysplasia for the prediction of progression if reliable protein markers were at hand.[13] In that case, high-risk lesions with limited dimensions could always be excised with larger surgical margins, while for patients with low-risk lesions watchful waiting with less frequent follow-up would suffice.

Because of the promising outlook that immunostaining approaches could be easily implemented, various studies have been carried out to identify protein biomarkers associated with malignant progression either using RNA-based[14] or, more recently, proteomics approaches.[15-22] In many studies proteins have been identified that display a decreased expression in tumors and/or severe dysplastic lesions. A large number of these

potential markers are associated with differentiation such as the cytokeratins that are part of the epithelial cytoskeleton, or the proteins forming other structures in differentiated keratinocytes such as the cornified envelope. Cornulin, keratin 4 and keratin 13 are often mentioned to be differentially expressed between normal mucosal epithelium and squamous tumor tissue of the oral cavity and have often been suggested as protein biomarkers that might be able to predict malignant progression,[14,15,17,18,23] but the clinical validation of this hypothesis is lacking. In a previous study using a differential proteomics approach, we also found that expression of cornulin, keratin 4 and keratin 13 is significantly decreased in tumors and severe dysplasia, preneoplastic tissue with a high risk of malignant progression, when compared to normal mucosa.[24] We further showed that decreased cornulin and keratin 4 expression in the resection margins of surgically treated head and neck cancer patients accurately predicted local relapse, while dysplasia grading failed. This indicates that these protein biomarkers might serve well to predict malignant transformation of premalignant mucosal changes in general. In the current study we investigated whether immunohistochemical analysis of the expression of these biomarker proteins is also of value to predict malignant transformation of leukoplakia lesions.

MATERIALS AND METHODS

Patients and leukoplakia lesions

In total 48 formalin-fixed paraffin-embedded biopsies of oral leukoplakia lesions of a consecutive series of 46 patients were included. Two patients had two clearly distinct leukoplakias that were considered separately. All biopsies of the leukoplakia lesions were histopathologically evaluated by an experienced pathologist (IvdW) according to the standard criteria of the World Health Organization.[25] The study was approved by the Institutional Review Board and in accordance to the Dutch guidelines on the use of human specimens for research.

Immunohistochemical analysis of cornulin, keratin 4 and keratin 13 expression

Sections of all leukoplakias were immunostained for cornulin, keratin 4 and keratin 13. The goat antibody against cornulin was from R&D Systems (Abingdon, UK) and used at a 1:200 dilution. The mouse monoclonal antibodies directed against keratin 4 and keratin 13 were from Abcam (Cambridge, UK) and used in dilution 1:100. All other antibodies were from DAKO (Glostrup, Denmark). Paraffin sections were deparaffinized, rehydrated, subjected to antigen retrieval by microwave boiling for 10 minutes in 10 mM TRIS pH 9.0, 1 mM EDTA, and pre-incubated for 15 minutes with 2% normal rabbit serum. Primary antibodies were

incubated for 1 hr. The second step was performed with a biotinylated rabbit-anti-mouse or rabbit-anti-goat antibody (1:500) and in the final step horseradish peroxidase labeled streptavidin-biotin-complex was applied. The staining was developed with diaminobenzidine and H_2O_2 as chromogen. The sections were counterstained with haematoxylin and coverslipped with Kaiser's glycerin. Positive (normal mucosa) and negative (tumor) controls were added with each immunohistochemical assay to ensure reproducibility of the immunostaining results. Mouse monoclonal antibody U36[26,27] and normal mouse IgG were included as positive and negative controls, respectively.

Semi-quantitative assessment of protein expression

Positive staining in the epithelial cells in the tissue sections was scored by multiplying the estimated percentage of stained cells (over the whole tissue section) by the staining intensity (0=absent, 1=weak or 2=strong) resulting in a score ranging between 0 and 200. For each biomarker protein, the leukoplakia lesions were divided into those with high (score: 100 to 200) and those with low (score: 0-99) expression. Kaplan-Meier curves were computed to analyze the relationships between protein biomarker expression and progression. Progression-free survival time was calculated as the time between the date of biopsy and the date of last follow-up visit or the date of histological diagnosis of malignant transformation. Log-rank tests were used to determine the significance of the associations. Student's T-tests and Fisher's Exact tests were used to detect confounding continuous or categorical variables, respectively. P-values <0.05 were considered significant.

RESULTS

Altered expression pattern of cornulin, keratin 4 and keratin 13 in leukoplakia

In normal epithelium, approximately 65%, 85% and 85% of the suprabasal cell layers displayed a homogeneous and strong (intensity 2) expression of cornulin, keratin 4 and keratin 13, respectively (Figure 1, first row). In tumor tissue the expression of these proteins could not be detected (Figure 1, second row). In leukoplakias, we found that in general the expression of all three proteins was decreased (Figure 1, third and last rows). Most striking example was keratin 4 of which no expression was detected in 42 out of 48 leukoplakias.

Hyperkeratosis, an aberrant or thickened keratinized layer of the mucosal epithelium, is a common phenomenon in leukoplakias. Strikingly, the epithelium underneath the hyperkeratosis often showed a very dissimilar staining pattern as compared to the epithelium without hyperkeratosis in the same leukoplakia biopsy (Figure 1). Therefore, we decided to score epithelium without hyperkeratosis and epithelium with hyperkeratosis

separately, and used the lowest score of expression, which is considered as most severe, as the final read-out for statistical analysis. Keratin 13 showed a very patchy expression pattern while it is highly and uniformly expressed in normal mucosa.

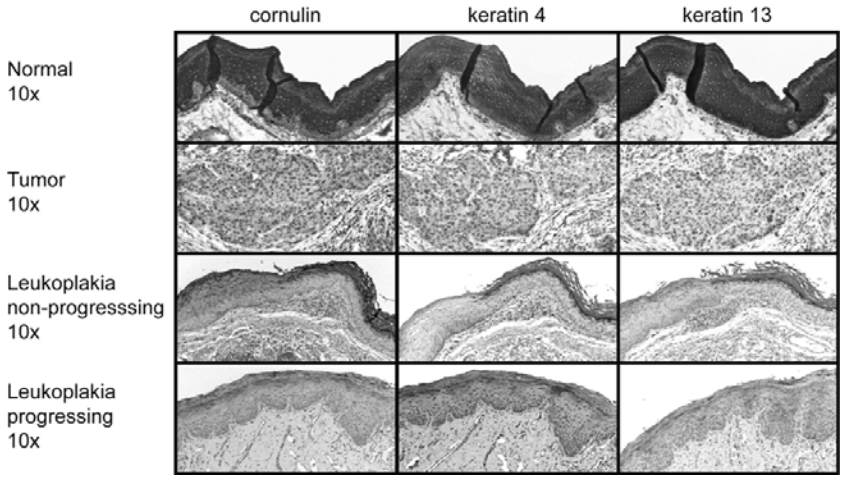

Figure 1. Examples of cornulin, keratin 4 and keratin 13 staining patterns in normal tissue, tumor tissue and leukoplakia lesions (available in color)

The most upper panels show the staining pattern of cornulin, keratin 4 and keratin 13 in normal mucosal epithelium and the second upper panels show the staining pattern in tumor tissue. The lower panels show the staining patterns in a non-progressing and progressing leukoplakia lesion. Note the irregular structure of the leukoplakia lesions with sharp demarcations of hyperkeratosis. These images further illustrate the heterogeneous and very diverse staining pattern of cornulin, keratin 4 and keratin 13 in leukoplakia lesions as compared to normal tissue.

Statistical analysis

Of the 48 leukoplakia lesions, twelve had progressed to cancer and 36 had not. The follow-up time ranged from 1 to 173 months with an average of 69 and a median of 61 months. Age (p=0.683), gender (p=0.497) or side of the lesion (p=1.0) were not associated with malignant progression. The presence of hyperkeratosis was also not associated with progression. We did not find any significant association between the calculated expression levels of cornulin, keratin 4 or keratin 13 and malignant progression (Figure 2). Only cornulin showed a trend (P=0.075). No stronger associations could be achieved by combining two or more parameters. Also, dividing the scores in quantiles did not improve statistical outcome.

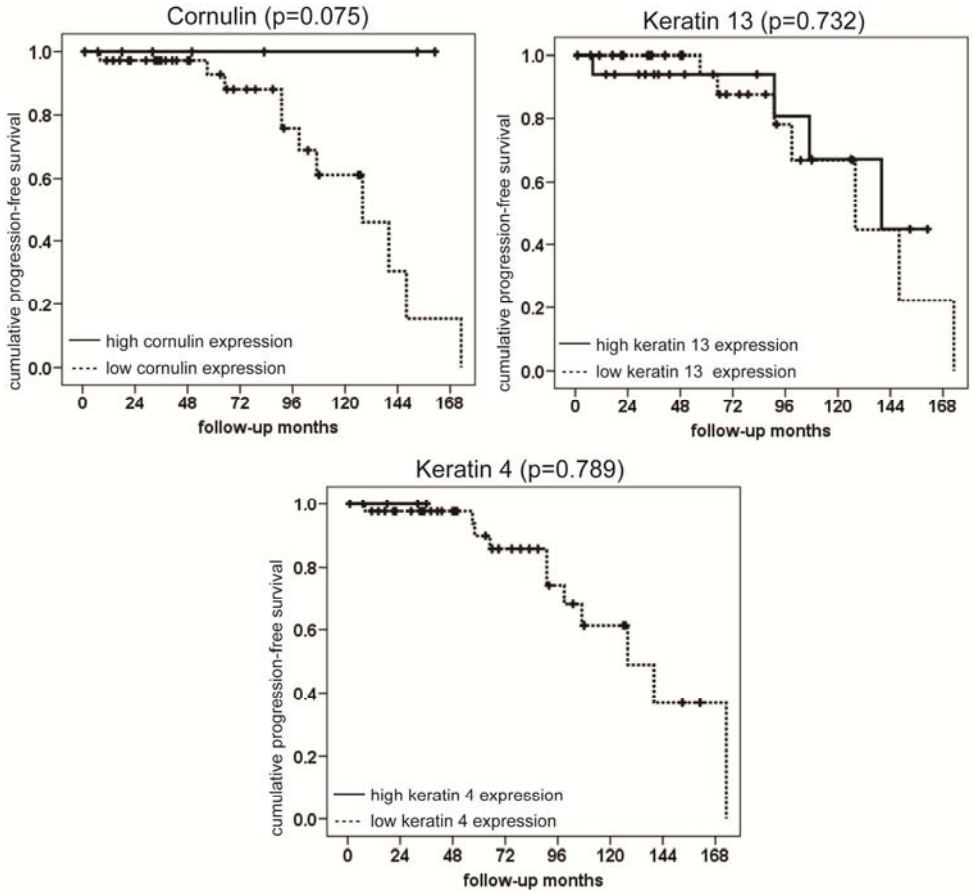

Figure 2. Kaplan-Meier curves of progression-free survival in relation to the expression of cornulin, keratin 4 and keratin 13

Kaplan-Meier curves of progression-free survival are depicted in relation to low cornulin, low keratin 4 and low keratin 13 expression. Protein expression was divided in two categories, low expression and high expression, the average of the possible staining score. Log-rank p-values are indicated above the graphs.

We did, however, find strong relationships between the presence of hyperkeratosis and low cornulin expression (p=0.001) and low keratin 13 expression (p=0.002). There was no association between hyperkeratosis and low keratin 4 expression. Finally we analyzed the association between dysplasia grading and malignant progression of the lesions. Lesions were categorized as either no (n=28), mild (n=7), moderate (n=7) or severe (n=6) dysplasia. Both the presence and grade of epithelial dysplasia was significantly associated with malignant transformation of leukoplakias (p=0.024 and p=0.029 respectively, Figure 3).

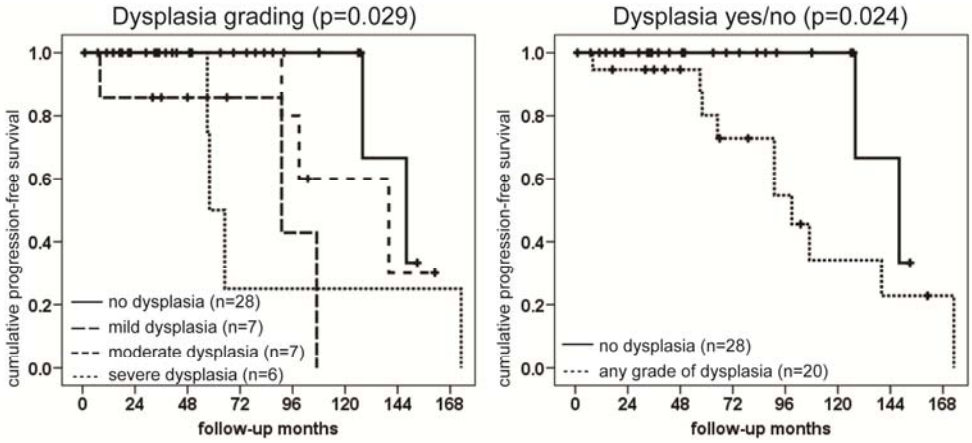

Figure 3. Kaplan-Meier curves of progression-free survival in relation to dysplasia grading

Dysplasia was divided in either four categories (no, mild, moderate, severe) or two categories (absence or presence of dysplasia). In both curves the association between dysplasia and the occurrence of a tumor are clear. Log-rank p-values are indicated above the graphs.

DISCUSSION

Oral leukoplakia is a quite common oral lesion, however, it is not always easily distinguished from other white lesions that might occur in the oral cavity. Recently, we found that expression of cornulin, keratin 4 and keratin 13 is significantly decreased in tumors and mucosal epithelium at high risk of malignant transformation, when compared to normal oral mucosa.[24] More importantly, we showed that decreased expression of cornulin and keratin 4 in the resection margins of surgically treated head and neck cancer patients accurately predicts local relapse, while dysplasia grading failed. It has been reported previously that cornulin, keratin 4 and keratin 13 show a decreased level of expression in oral tumors and leukoplakia lesions[14,15,17,18,23,28,29], but none of these studies focused on the association with progression. Therefore, we decided to determine the value of these proteins to predict the risk of progression of leukoplakia lesions.

Here we report that altered expression of cornulin, keratin 4 and keratin 13 in leukoplakia lesions does not show any significant association with progression. An likely reason for this is that expression of these three proteins is already tremendously decreased in all leukoplakia lesions, irrespective of the risk of malignant progression. Cornulin, keratin 4 and keratin 13 are involved in differentiation and this might well explain why the expression appears to be dysregulated in leukoplakia. Oral leukoplakias are often characterized by hyperkeratosis, indicating an aberrant, more skin-like, histological differentiation. We found that hyperkeratosis is significantly associated with the loss of expression of at least two of these three proteins. All proteins also displayed a very heterogeneous expression pattern, which seems to be related to the irregular tissue structure of leukoplakias with areas with and without hyperkeratosis. This heterogeneity also hampered a reliable semi-quantitative scoring method for the immunostained sections of these lesions. We conclude that the change in expression of these proteins mainly results from the aberrant tissue differentiation of leukoplakia lesions, which obscures decreased expression in relation to malignant progression. Of note, the currently used sample size was relatively small. Nevertheless, the very high p-values indicate that increasing the sample size will not result in strong associations between decreased keratin 4 and/or keratin 13 expression and malignant transformation. It might, however, be worthwhile to evaluate the potential of cornulin in a larger cohort of patients, as it already showed a trend in this study. In our view, protein biomarkers not associated with mucosal dedifferentiation might be more promising candidates.

Since dysplasia grading is the current method of choice to predict malignant transformation of leukoplakias, we decided to test its value in our patient cohort. We noted that the

presence of dysplasia was significantly associated with malignant progression (p=0.024; Figure 3). This is discordant with the results reported by Holmstrup *et al.* who did not find a significant relationship between epithelial dysplasia and progression in a larger cohort of 269 leukoplakias.[30] When adjusting for the erythroplakia lesions included in the study by Holmstrup *et al.* and correcting for the biopsies that were missing, they found dysplasia in 39% of all evaluable leukoplakia cases while we observed a comparable 42%, indicating that the cohorts seemed comparable at least with respect to the frequency of dysplasia. Multicenter investigations with large patient cohorts and observers from different institutes might shed more light on the significance of dysplasia grading for the prediction of malignant transformation. Based on our data, immunostaining using differentiation markers does not seem to be of significant importance in this respect, despite that these might be helpful in other applications as reported.

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Chapter 5

Decreased expression of keratin 4 and cornulin in surgical margins predicts local relapse in head and neck cancer patients

Tieneke B. M. Schaaij-Visser
Boudewijn J. M. Braakhuis
Marijke Buijze
Albert J. R. Heck
Dirk J. Kuik
Elisabeth Bloemena
C. René Leemans
Monique Slijper
Ruud H. Brakenhoff

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ABSTRACT

The 5-year-survival rates of head and neck squamous cell carcinomas (HNSCCs) remain disappointing. HNSCCs develop in precursor fields of genetically altered cells that are often not completely resected when the tumor is excised, causing local relapse. These precursor fields are mostly recognized as dysplasia, but histological grading cannot reliably predict malignant transformation. Recently, we identified cornulin and keratin 4 as promising protein biomarkers to identify precursor fields. Here, we evaluate in a retrospective case-control study the potential of these protein biomarkers to predict local relapse of HNSCC by immunostaining of the surgical margins.

The prognostic value of keratin 4 and cornulin expression was evaluated by immunohistochemical analysis of 222 surgical margins of 46 HNSCC patients who developed local relapse or remained disease-free. Significant associations were determined by Kaplan-Meier survival analysis and Cox-proportional-hazards-models. We found that low expression of keratin 4 ($p=0.002$, hazard ratio=3.8), cornulin ($p=0.025$, hazard ratio=2.7), and their combination ($p=0.0005$, hazard ratio=8.8) in the surgical margins showed a highly significant association with the development of local relapse. Dysplasia grading had no prognostic relevance. We conclude that immunohistochemical assessment of keratin 4 and cornulin expression in surgical margins of HNSCC patients outperforms histopathological grading in predicting the risk of local relapse. These markers can be used to initiate more frequent and lifelong surveillance of patients at high risk of local relapse, and enable selection for adjuvant treatment or secondary prevention trials.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) develops in the mucosal linings of the upper aero-digestive tract and is the sixth most common cancer worldwide.[1] The five-year-survival rates of HNSCC are approximately 60%[1] and have only moderately improved the last decades[2] mainly because 20-40% of all patients develop a local relapse in the same or adjacent anatomical region even when the surgical margins are histologically tumor-free.[3, 4] Clinically these relapses at the primary and adjacent anatomical sites are assigned as local recurrences when they develop within three years and at < 2 cm distance of the primary tumor. Relapses not fulfilling these criteria are clinically classified as second primary tumors (SPT). Despite the different clinical assignment many local relapses have in fact the same pathobiological origin.[3-8]

It has been well established that HNSCC is the result of a multistep process characterized by the accumulation of genetic and epigenetic alterations.[9] Genetic analysis of surgical margins has shown that HNSCC frequently develops in a field of genetically altered epithelial cells that are referred to as precursor fields.[10, 11] These fields can reach dimensions of several centimetres in size without any clinical symptoms and the large majority of these fields cannot be recognized by the naked eye. Very recently, however, it has become possible to visualize these fields using autofluorescence, which apparently confirms their existence and dimensions.[12, 13] Only a minority of the precursor fields might appear as clinically identifiable lesions that present as either white or red mucosal areas: leukoplakia and erythroplakia, respectively. In the precursor fields a tumor can develop that will be diagnosed and often surgically treated. However, the fields are not always resected entirely when the primary tumor is excised. Malignant transformation of an unresected precursor field in a patient might cause a local relapse that is clonally related with the field and the index tumor.[5-8, 14] Treatment of these relapses is difficult as they occur in tissues affected by previous treatment and occasionally present at an advanced stage. Outcome might be greatly improved by early diagnosis of these local relapses through frequent surveillance of patients that harbour precursor fields.[15] Hence, when these patients could be identified they should remain under lifelong surveillance or receive adequate adjuvant treatment to eradicate these fields.

When HNSCC patients are surgically treated, the margins of the excised specimens are routinely investigated by histopathological examination for the presence of residual cancer as well as unresected precursor fields that are usually recognized as dysplastic mucosal changes. Histopathological grading of dysplasia is the gold standard for identification and risk assessment of precursor fields in surgical margins, but it is somewhat subjective and

has limited value to predict local relapse in individual cases. Only severe dysplasia might be considered an indication for adjuvant post-operative radiotherapy, but not as a single criterion. Alternatively, genetic markers such as loss of heterozygosity and mutated p53 can be determined in DNA isolated from the surgical margins, and these have been reported to predict local relapse.[6, 14, 16] However, the prognostic value of these approaches is still limited and the required methods are laborious and not easy to implement in current clinical management.

In Chapter 3 was described how we identified a panel of novel candidate protein biomarkers that might allow prediction of local relapse using differential proteome analysis of paired and genetically characterized normal, precursor and tumor tissues. Here, we validate the two most promising protein biomarkers, keratin 4 and cornulin, in a retrospective case-control study by immunostaining of the surgical margins of surgically treated head and neck cancer patients who either developed a local relapse or remained local disease-free.

MATERIALS AND METHODS

Tissue samples

After tumor excision the surgical margins are routinely removed from the specimen, formalin-fixed, and paraffin-embedded (FFPE) for histological examination by the pathologist. These FFPE surgical margins were used for this study. HNSCC patients (n=46) were included, who either developed a local relapse during follow-up in the same or adjacent anatomical region, or remained disease-free. Further criteria for inclusion were: 1) primary tumor in the oral cavity or oropharynx, 2) surgical treatment with >5 mm histologically tumor-free surgical margins, and 3) N-stage ≤N2b. Cases and controls were matched for primary treatment (surgery with/without postoperative radiotherapy) and T-stage (T1/2 or T3/4). For comparison to the current standard, every surgical margin was graded for dysplastic changes as either no [0], mild [1], moderate [2] or severe [3] dysplasia. As final readout, the highest grade of dysplasia scored in any of the surgical margins of a patient was taken.

The study was approved by the Institutional Review Board and in accordance to the Dutch guidelines on the use of human specimens for research. All histological evaluations and dysplasia grading were performed by an experienced pathologist (E.B.) according to the standard criteria of the World Health Organization.[17]

Immunohistochemistry

The monoclonal mouse antibody directed against keratin 4 was from Abcam (Cambridge, UK) and used in dilution 1:100. The goat antibody against cornulin was from R&D Systems (Abingdon, UK) and used 1:200. All other antibodies were from DAKO (Glostrup, Denmark). Paraffin sections were deparaffinized, rehydrated, subjected to antigen retrieval by microwave boiling for 10 minutes in 10 mM TRIS pH 9.0, 1 mM EDTA, and pre-incubated for 15 minutes with 2% normal rabbit serum. Primary antibodies were incubated for 1 hr. The second step was performed with a biotinylated rabbit-anti-mouse or rabbit-anti-goat antibody (1:500) and in the final step horseradish peroxidase labeled streptavidin-biotin-complex was applied. The staining was developed with diaminobenzidine and H_2O_2 as chromogen. The sections were counterstained with haematoxylin and coverslipped with Kaiser's glycerin. Positive (normal mucosa) and negative (tumor) controls were added with each immunohistochemical assay to ensure reproducibility of the antibodies. Mouse monoclonal antibody U36 and normal mouse IgG were included as positive and negative controls, respectively.

Semi-quantitative assessment of protein expression

In total 222 surgical margins of 46 patients with known follow-up were immunohistochemically stained for keratin 4 and cornulin. The level of protein expression in the immunostained samples was recorded by two independent observers who were blinded for the clinical outcome data, and defined as the percentage of positive cells. After independent scoring, the results were compared and a final consensus-score was appointed for each surgical margin. The overall score that was used for statistical analysis in the prognostic study was the averaged mean of all the surgical margins of that particular patient. To enable statistical analysis for ordinal data, percentages were categorized as either high (above median) or low (below median). In order to explore the prognostic value of the combination of decreased keratin 4 and cornulin expression, the categorical values for both proteins were combined to make a division between normal (high keratin 4 and high cornulin) and aberrant (low keratin 4 and/or low cornulin) protein expression.

Statistical analysis

Local relapse-free survival time was calculated from the date of histological diagnosis of the primary tumor to the date of local relapse or the date of last follow-up. Kaplan-Meier curves were computed to assess local relapse-free survival[18] and the log-rank test was performed to determine the difference between the curves.[19] Variables that showed significant relationships with local relapse-free survival in the univariate models were included in a

multivariate Cox proportional-hazard model to determine associations.[20] Dysplasia in the surgical margins was included in the statistical analysis, both divided in four (no, mild, moderate and severe) and in two (no/mild and moderate/severe) categories. ROC curves were calculated with the original averaged staining percentages to explore the sensitivity and specificity of the immunostaining for the respective biomarkers. The SPSS 15.0.1 statistical package was used for all analyses, and p-values of less than 0.05 were considered statistically significant.

RESULTS

Study cohort

Clinicopathological characteristics of the case and control group are summarized in Table 1.

Table 1. Clinicopathological characteristics of the patients

	Disease-free	*Local relapse*	*P-value*[#]
Total number	23	23	
Gender[1]			
Male	14	15	1.00
Female	9	8	
Average age[2]	57	57	0.85
Tumor site[1]			
Oral cavity	19	17	0.72
Oropharynx	4	6	
N-status[1]			
N0	12	11	1.00
N+	11	12	
Average # margins[2]	4.6	5.1	0.17
Dysplasia margins[1]			
No	8	9	1.00
Mild	7	6	1.00
Moderate	4	4	1.00
Severe	4	4	1.00
Any grade	15	14	1.00

[#] Fisher's Exact for categorical variables[1] and t-test for continuous variables[2]

Our study cohort consisted of 29 men and 17 women with an average age of 57.0 years (range 38 to 86) at the time of diagnosis. In total 23 patients had tumor-free lymph nodes, while 23 patients showed lymph node metastases. Twenty-two patients were treated by surgery only and the remaining received adjuvant radiotherapy. The median local disease-free survival time was 5.4 years (range 0.5 to 13.9), during which the 23 case patients developed a local recurrence (n=19) or second primary tumor (n=4). For every patient, the surgical margins were divided over 2 to 7 separate tissue blocks, which added up to a total of 222 different surgical margins. Table 2 lists all the individual patients and their characteristics.

Table 2. Patient characteristics

pt	gender	age	site	TN-stage	treatment	local follow-up	follow-up time	# of margins	dysplasia margins	score keratin 4	score cornulin
1	m	68	oral cavity	T1N0	surg	df	6,3	4	moderate	23	47
2	m	58	oropharynx	T1N0	surg	loc	0,7	4	no	41	62
3	m	65	oral cavity	T1N0	surg	df	5,5	3	moderate	54	60
4	f	86	oral cavity	T1N0	surg	loc	2,4	4	no	31	46
5	m	71	oral cavity	T1N0	surg	df	4,8	4	no	69	64
6	m	48	oral cavity	T1N0	surg	spt	7,6	4	mild	50	44
7	m	39	oral cavity	T1N0	surg	df	12,0	5	no	67	65
8	m	63	oral cavity	T1N0	surg	loc	1,6	4	moderate	7	42
9	m	47	oral cavity	T1N0	surg	df	9,9	6	severe	48	63
10	m	67	oral cavity	T1N0	surg	loc	1,5	3	mild	6	46
11	f	49	oral cavity	T1N1	surg	df	13,9	3	mild	48	59
12	m	56	oral cavity	T1N0	surg	spt	3,9	5	moderate	38	40
13	m	58	oral cavity	T2N1	surg	df	4,4	7	mild	25	50
14	m	62	oral cavity	T2N1	surg	loc	1,0	6	no	18	38
15	f	54	oral cavity	T2N1	surg	df	8,5	4	mild	44	53
16	f	67	oral cavity	T1N0	surg	spt	6,1	3	moderate	34	44
17	f	56	oral cavity	T2N1	surg	df	12,2	4	no	58	60
18	m	38	oral cavity	T2N0	surg	loc	1,8	4	moderate	30	43
19	m	53	oral cavity	T2N2	surg	df	12,0	7	moderate	46	51
20	f	53	oral cavity	T3N1	surg	loc	1,4	5	mild	32	62
21	m	59	oral cavity	T4N0	surg	df	5,3	5	no	65	61
22	m	72	oral cavity	T3N2	surg	loc	0,5	6	no	34	50
23	f	54	oral cavity	T2N0	surg+rt	df	9,6	7	mild	27	49
24	m	40	oropharynx	T1N2	surg+rt	loc	1,5	3	mild	40	59
25	f	66	oral cavity	T2N0	surg+rt	df	7,4	4	no	62	58
26	f	59	oral cavity	T1N0	surg+rt	spt	0,5	2	severe	36	53
27	m	63	oral cavity	T2N2	surg+rt	df	9,2	5	mild	66	57
28	m	47	oral cavity	T2N2	surg+rt	loc	0,5	5	no	43	45
29	m	51	oropharynx	T2N2	surg+rt	df	11,2	6	moderate	54	58
30	f	66	oral cavity	T2N0	surg+rt	loc	1,6	4	mild	27	41
31	f	58	oral cavity	T2N2	surg+rt	df	6,2	5	no	35	48
32	m	60	oral cavity	T2N2	surg+rt	loc	1,1	5	mild	11	41
33	m	43	oral cavity	T2N2	surg+rt	df	11,2	4	severe	49	51
34	m	43	oral cavity	T2N2	surg+rt	loc	0,5	7	no	43	43
35	f	71	oropharynx	T3N0	surg+rt	df	7,8	5	mild	48	39
36	m	51	oropharynx	T3N2	surg+rt	loc	3,3	5	no	54	64
37	m	69	oral cavity	T3N0	surg+rt	df	10,0	6	no	53	49
38	m	51	oropharynx	T3N2	surg+rt	loc	2,0	4	severe	27	49
39	f	53	oropharynx	T3N0	surg+rt	df	7,6	6	severe	60	43
40	f	56	oropharynx	T3N1	surg+rt	loc	1,8	5	no	46	41
41	m	55	oral cavity	T3N2	surg+rt	df	9,5	7	no	68	62
42	f	46	oropharynx	T4N0	surg+rt	loc	3,0	4	severe	53	40
43	f	69	oral cavity	T4N0	surg+rt	df	6,9	5	mild	31	28
44	m	50	oral cavity	T4N2	surg+rt	loc	0,7	7	severe	60	59
45	m	46	oropharynx	T4N1	surg+rt	df	11,2	5	severe	33	56
46	f	65	oral cavity	T4N2	surg+rt	loc	0,9	6	no	12	47

Note: pt=patient number, m=male, f=female, surg=surgery, surg+rt=surgery and radiotherapy, loc=local recurrence, spt=second primary tumor, df=disease-free

Semi-quantitative scoring

All 222 margins were immunostained and independently evaluated by two observers. Overall scores for all individual patients are provided in Table 2. Figure 1 illustrates the high inter-observer accuracy of the quantitative evaluation of keratin 4 and cornulin expression with correlation coefficients of 0.92 and 0.87, respectively. Examples of the staining patterns are shown in Figure 2, which represents the images of one patient who remained disease-free and one patient who developed a local relapse.

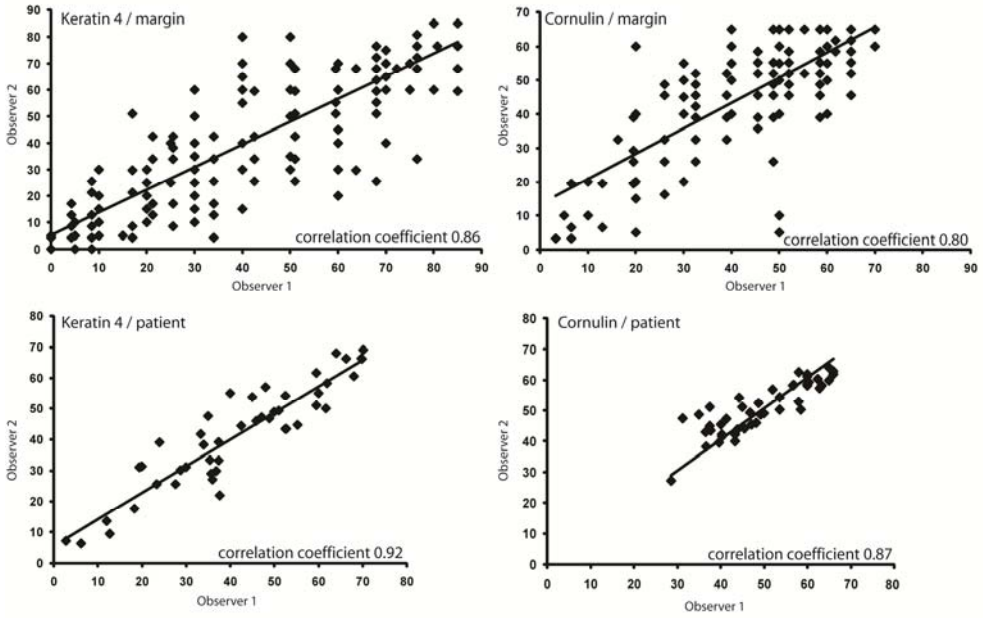

Figure 1. Inter-observer correlations of semi-quantitative scoring

Correlations between quantitative assessments of the staining results of observer 1 with observer 2. The upper panel shows the correlation of the assessment of the individual margins, while the lower panel shows the results on the averaged percentages for every patient. The high correlation coefficients demonstrate the accuracy of the quantitative scoring.

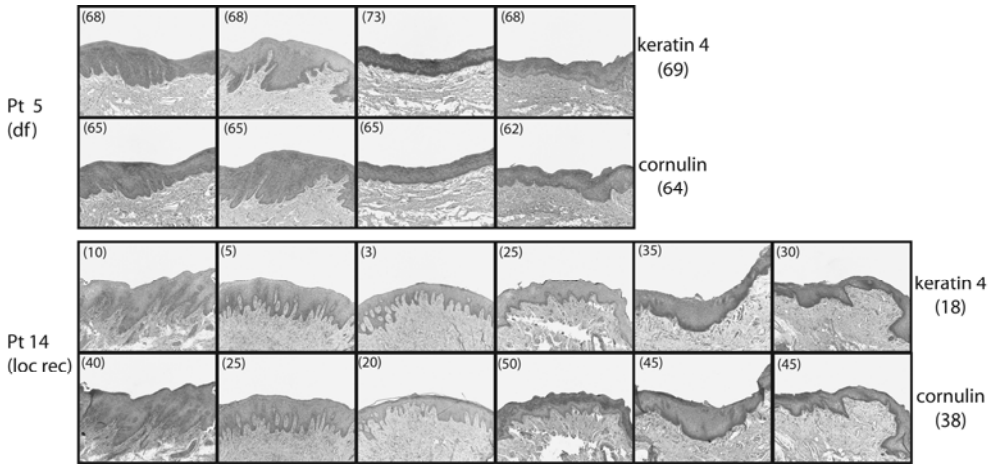

Figure 2. Examples of immunostaining of surgical margins (available in color)

Representative images of immunostained formalin-fixed paraffin-embedded surgical margins of two patients; one who remained disease-free (df; pt 5) and one who developed a local relapse (loc rec; pt 14). Consensus-score for every margin of both keratin 4 and cornulin is indicated between brackets. Staining patterns can vary slightly throughout one margin. Averaged staining percentages per patient are given between brackets at the right-hand side of the images.

Statistics

The median of the averaged scores of all patients was used to divide the samples into two categories, one with high expression and one with low expression. For keratin 4 the median was 43.0% and for cornulin 49.5%. Kaplan-Meier curves and the log-rank test revealed that the development of local relapse was significantly associated with a low expression of keratin 4 and cornulin in the surgical margins of the index tumor as depicted in Figure 3. Hazard ratios calculated by univariate Cox regression analysis, were 3.8 (p=0.004, 95% confidence interval 1.6-9.5) for keratin 4 and 2.7 (p=0.031, 95% confidence interval 1.1-6.5) for cornulin.

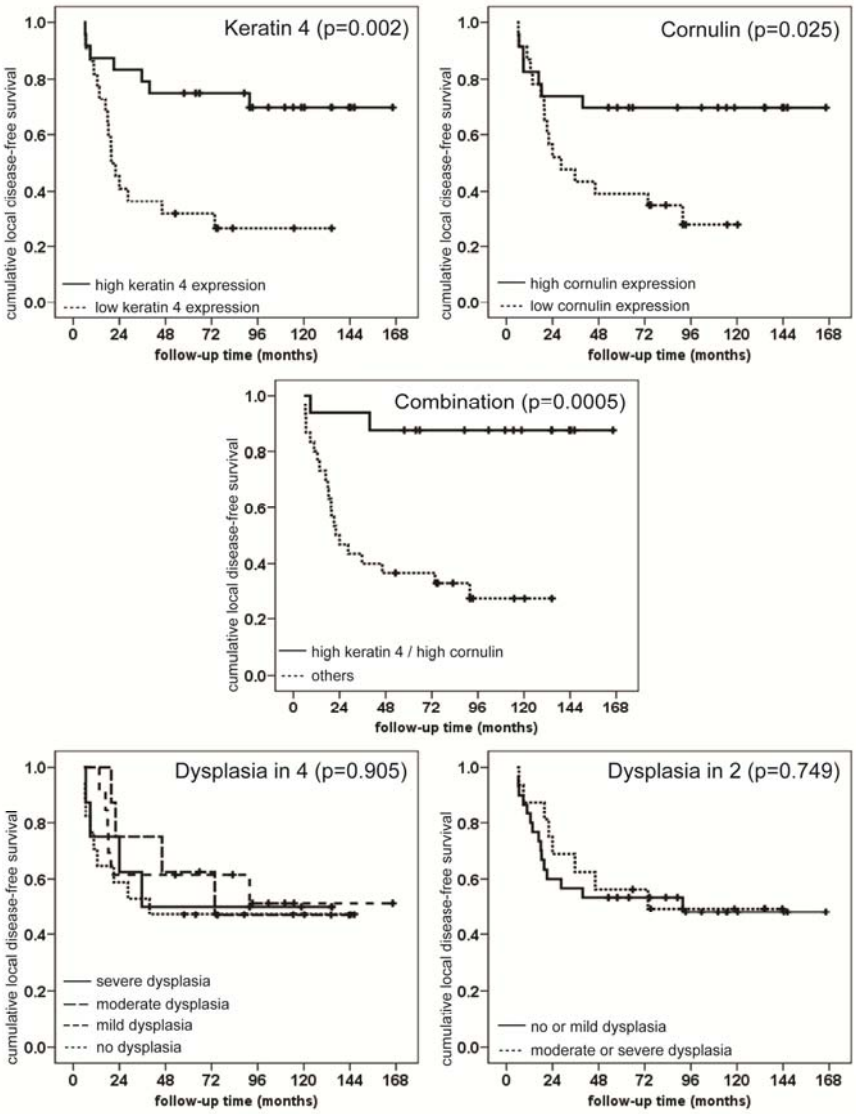

Figure 3. Kaplan-Meier curves of local relapse-free survival in relation to immunostaining and histological grading of surgical margins

In the upper panels curves are depicted in relation to a low keratin 4 expression, low cornulin expression, or the combination. Averaged percentages for protein expression were divided in two categories, low expression (below median) and high expression (above median). For the combined analysis, patients with normal expression patterns (high keratin 4 and high cornulin expression) were grouped together and patients with aberrant expression patterns (low keratin 4 and/or low cornulin expression) were grouped together. In the lower two graphs the Kaplan-Meier survival curves according to dysplasia grading are depicted. Dysplasia was divided in either four categories (no, mild, moderate, severe) or two categories (no/mild, moderate/severe). Log-rank p-values are indicated within the graphs.

The keratin 4 and cornulin immunostaining data were combined to form one group of patients with high keratin 4 and high cornulin expression in the surgical margins, reflecting a normal staining pattern, and another group containing all other patients (low keratin 4 and/or low cornulin), reflecting an aberrant staining pattern. This classification showed a very strong association with local relapse-free survival (p=0.0005) and has a superior prognostic power with a hazard ratio of 8.8 (p=0.004, 95% confidence interval 2.0-37.6). Grade of dysplasia in the surgical margins did not show a significant association with local relapse-free survival (Figure 3) neither when categorized in 4 classes (no, mild, moderate or severe) nor in two classes (no/mild versus moderate/severe).

ROC curves, based on the continuous averaged percentage data, were computed to explore sensitivity and specificity of the individual markers, displaying an AUC of 0.77 for keratin 4 and 0.71 for cornulin Figure 4.

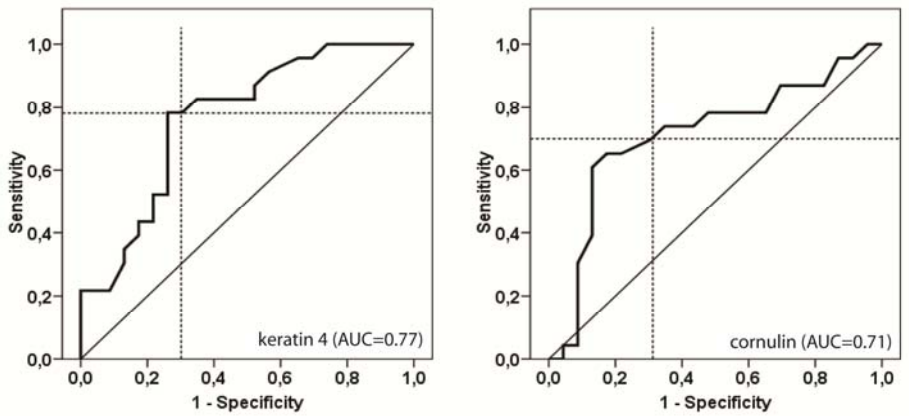

Figure 4. ROC curves for keratin 4 and cornulin

ROC curves representing the relation between sensitivity and specificity for keratin 4 and cornulin immunostaining in the prognostic validation study. Curves were computed based on averaged staining percentages. Significant (p<0.05) areas under the curve (AUC) are indicated within the graph. Dashed lines specify the medians, which were used to categorize the patients into those with high and low keratin-4 and cornulin expression levels.

DISCUSSION

Clinical management of head and neck cancer is greatly hindered by the large proportion of patients that develop a local relapse in the same or adjacent anatomical region. Histological grading of the mucosal epithelium is still the gold standard to assess the risk of malignant progression but meets several limitations. A simple straightforward immunostaining procedure on the surgical margins of surgically treated HNSCC patients would be an ideal diagnostic tool to predict local relapse if protein biomarkers were available. For this study we clinically validated two potential biomarkers identified as described in Chapter 3, in a retrospective case-control study.

We demonstrated that low expression of keratin 4 and cornulin in the resection margins of surgically treated HNSCC patients accurately predicts local relapse and outperforms histopathological grading. An explanation for this better performance may be that the immunostaining is scored semi-quantitatively, which is more objective and reproducible. The subjectivity and intra- and interobserver variability forms a major obstacle in dysplasia grading. The semi-quantitative scoring of immunostaining might also tackle the heterogeneity in morphology of the mucosa from different locations within the head and neck region, which also might hinder accurate dysplasia grading. Still, the accuracy is also protein biomarker-specific. Keratin 4 showed a much better prognostic performance than keratin 13 (also described in Chapter 3 and analyzed on a subset of samples: data not shown), and also seemed better when compared to cornulin. This could be explained by its broad range of expression which is illustrated in Figure 1 allowing a larger differential power, a more reliable assessment of the immunostained sections, and a higher correlation between observers.

Although it is known that the expression of keratin 4 and cornulin, like many others, change during the carcinogenic process[21-28], no studies have been published that specifically elaborate on the prognostic value of these proteins to predict local relapses when analyzed on the resection margins of surgically treated HNSCC patients. Due to our stringent inclusion criteria, a relative small number of patients was included. Nevertheless, the statistical outcomes were highly significant; in particular when the markers were combined in a “normal” (both high) and “aberrant” (one or both low) staining pattern. For this study, we used the (unbiased) median expression to define high and low expression; for clinical applications a cut-off could be set based on the desired sensitivity and specificity (see Figure 4).

Keratin 4 and cornulin immunostaining of the surgical margins might be used in the decision-making process for post-operative radiotherapy and to determine the surveillance policy for

treated HNSCC patients during follow-up. Depending on the medical center, the follow-up surveillance usually encompasses a visit every 6 to 8 weeks during the first year, gradually decreasing to once per year and then stops at five years. Patients with apparent unresected precancerous fields, indicated by low keratin 4 and cornulin expression in the surgical margins, should remain under long, and perhaps lifelong, frequent surveillance, while for patients without these fields, surveillance could remain the same as applied at present. Further, loss of keratin 4 and cornulin expression seems a valuable enrolment criterion for tertiary prevention trials in treated HNSCC patients. Several experimental approaches are being studied and showed promising initial results.[29-31]

Our approach can be easily implemented for patients scheduled for tumor resection as surgical margins are routinely obtained from the excised specimen. It remains to be determined whether it could also be exploited for analysis of multiple biopsies taken around the tumor when patients are scheduled for chemoradiation. As the number of biopsies that can be taken is limited, sampling error might easily cause false-negative results. The first recent successes in visualizing these precursor fields by autofluorescence might turn out to be a great aid to select the mucosal regions at risk[12, 13] in these patients. Hence, these visualization tools may be a suitable approach when used in combination with biopsy and keratin 4/cornulin immunostaining for the identification of patients with fields who are scheduled for treatment regimens without surgery.

Taken together, our results show that keratin 4 and cornulin, evaluated by a simple immunostaining on routine FFPE surgical margin specimens, seem to have large prognostic value to predict local relapse. A large-scale prospective multi-center trial will have to be carried out to definitively establish the value of this approach in the clinic.

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Chapter 6

An overview of protein biomarker discovery for head and neck cancer

Tieneke B. M. Schaaij-Visser
Ruud H. Brakenhoff
C. René Leemans
Albert J. R. Heck
Monique Slijper

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ABSTRACT

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer world-wide. Despite improvements in diagnosis and treatment, the five-year-survival rate of advanced HNSCC has only moderately increased, which is largely due to the high proportion of patients that present with advanced disease stage and the frequent development of relapse and second primary tumors. Protein biomarkers allowing early detection of primary HNSCC or relapse may aid improved clinical outcome. To predict risk of malignant transformation, screening for precursor changes in the mucosal linings preceding the development of invasive tumors may be a propitious opportunity, which is as yet impossible. This review summarizes recent results in HNSCC proteomics for biomarker research. Despite the wide diversity of experimental designs, a few common markers have been detected. Although some of these potential biomarkers are very promising, they still have to be further clinically validated. Finally, treatment of advanced cancers of several sites within the head and neck has shifted significantly during the last decade, and also, targeted drugs have entered the clinic. This has major consequences for the research questions in head and neck cancer research and accordingly for the future direction of proteome research in HNSCC biomarker discovery.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises all tumors that arise in the squamous epithelium aligning the oral cavity, pharynx, larynx and cervical esophagus. Worldwide, HNSCC accounts for 6% of all cancer cases and forms the sixth cause of cancer related death.[1] HNSCCs arise mostly between the 6th and 7th decade of life.[1] Although typically men are developing HNSCC, the incidence of the disease is rising amongst both young people and woman, most likely due to changing lifestyles.[2] The overall 5-year-survival rates vary around 50%, depending largely on the tumor stage at diagnosis.

The main causes of HNSCC are smoking and excessive consumption of alcoholic beverages that have together a synergistic effect.[3-8] Recently, infection with the sexually transmitted human papilloma virus (HPV) was found to be involved in HNSCC development as a new etiologic factor, particularly in oropharyngeal cancer.[9-16] HPVs are strictly epitheliotrophic DNA viruses and the known cause of cervical cancer. There are more than 100 subtypes distinguished of which particularly HPV type 16 and 18 account for over 70% of all cervical carcinomas. High-risk HPVs produce two oncoproteins, encoded by the E6 and E7 genes, that inactivate the p53 and pRb proteins, respectively. The most commonly detected HPV-type in HNSCC is the high-risk type HPV16.[17-20]

Even though tobacco smoking and heavy alcohol consumption are the classical risk factors, the large majority of smokers and alcohol abusers do not develop HNSCC, thus besides exposure also genetic factors render some individuals more sensitive for these environmental factors than others.[21, 22] This is most prominently observed in patients with an inherited genetic predisposition for HNSCC such as patients with Fanconi Anaemia (FA). FA patients have a mutation in the genes involved in the FA/BRCA pathway, which plays a role in maintenance of DNA stability. These patients show congenital abnormalities and bone marrow failure, but are also cancer prone. They frequently develop acute myeloid leukemias, and have a relative risk of 500-1000 times to develop squamous cell carcinomas, predominantly in the oral cavity, which occurs already at early age. Besides severe genetic disorders such as FA that have a major impact on cancer risk, there are several genetic variations in the enzymes responsible for metabolism and detoxification of the carcinogenic substances in cigarette smoke and alcohol that also cause a moderately increased cancer risk.[23-25] In addition, it has been reported that 10% of the population shows an increased sensitivity to mutagenic agents, which correlates with an increased risk for HNSCC development.[26, 27] Hence, HNSCC is a classical example of a tumor that arises through the interplay of genetic and environmental risk factors.

The dilemma of early detection and recurrence of HNSCC

Treatment of HNSCC patients focuses on the primary tumor and the neck.[28] HNSCCs preferentially metastasize via the lymphatic route to the lymph nodes in the neck, and when lymph node metastases are detected, the neck should be treated. The presence of lymph node metastases in the neck is also the most important prognostic factor for HNSCC. This includes not only the presence of lymph node metastases, but also the level in the neck, the number of involved nodes and the presence of extranodal spread. A problem is that occult metastases may be present in the clinically negative (cN0) neck, causing a difficult decision problem for the treating clinician whether to follow a watchful waiting policy or elective treatment of the neck. It is well-known that approximately 30 to 40% of the patients with a clinically N0 neck may harbor lymph node metastases, and methods to improve the neck staging are urgently awaited.

Patients with early stage tumors (stage I or II) without nodal metastases are treated by surgery or radiotherapy and have excellent five-year-survival rates. Unfortunately, two thirds of all patients present with advanced stage III or IV tumors, which is due to the fact that the large majority of HNSCCs arises *de novo*, without preceding visible changes in the mucosa, and also, there is often a delay in seeking medical treatment. In addition, squamous cancers progress from early to advanced stage within weeks, which also hampers early diagnosis of HNSSCs.

Patients with advanced stage tumors are treated by surgery, followed by radiotherapy or chemo-radiation, which often leads to significant functional impairment in swallowing and speech, while their five-year-survival rates remain below 60%. During the last decades, progress has been made in the development of novel treatment modalities for HNSCC.[28-30] Although these developments have resulted in improved locoregional control and reduced treatment-related morbidity, five-year-survival rates have not improved accordingly.[31] Hence, early diagnosis of primary tumors seems to remain key to improve treatment outcome.

A major problem in the clinical management of HNSCCs is the frequent occurrence of tumor relapses in the same or adjacent anatomical region, even when the surgical margins were tumor-free as investigated by the pathologist through histology. These relapses are clinically assigned as local recurrence when they develop within three years and at < 2 cm distance of the primary tumor.[32] Relapses not fulfilling these criteria are clinically classified as second primary tumors.[33, 34] Salvage treatment of these relapses is often difficult, since they occur in tissues affected by previous treatment.

A second and increasingly important clinical problem is the development of distant metastases, that may occur in 10-25% of the HNSCC patients. The primary sites for distant metastases are the lungs, the liver and the skeleton, for which at present no effective

systemic treatment exists, while the presence of distant metastases usually leads to disease-related death. Finally, as mentioned above, HNSCC patients also develop second primary tumors, not only in the same or adjacent anatomical regions, but frequently they also develop as non small cell lung cancer (NSCLC). This is to be expected, as primary lung cancer is caused by the same interplay between genetic and environmental factors (tobacco smoke exposure) as HNSCC. The frequent occurrence of either a distant metastasis or a second primary NSCLC causes a serious problem of differential diagnosis of a pulmonary lesion, that may affect the clinical management of these patients.

HNSCC carcinogenesis and precancerous fields

As for all cancers, it has been well established that HNSCC is the result of a multistep process, characterized by the accumulation of genetic and epigenetic alterations, causing activation of oncogenes and inactivation of tumor suppressor genes.[35] More than a decade ago, it was shown that the accumulation of particular genetic changes is associated with increasing histological abnormalities, and the first genetic progression model for HNSCC was presented.[36] The basis of this model, however, was still histological grading, and a few years ago Braakhuis *et al.*[37] adapted the molecular progression model for HNSCC carcinogenesis. This improved model indicates that HNSCCs arise in precancerous fields of genetically altered epithelial cells, also named precursor fields. These precursor fields are often characterized by p53 pathway disruption[38-40] and by allelic losses at chromosome arms 3p, 9p and 17p[41-43]. These fields can reach dimensions of up to several centimeters in diameter without any clinical symptoms such as pain. Additionally, the large majority is not visible to the naked eye, just a subgroup is clinically identifiable as white (leukoplakia) or red (erythroplakia) plaques in the mucosa. The pathologist usually recognizes precancerous fields as dysplasia under the microscope, grading them as mild, moderate or severe. A problem is, however, that this histological grading is somewhat subjective and that not all precancerous changes are recognized, which hampers the clinical management of these precancerous fields. Within such a precursor field a tumor can develop that will be diagnosed in the course of the disease. As these fields are generally large and not visible to the naked eye, they are often not completely resected when a tumor is excised. Histological grading of the surgical margins does not allow reliable prediction of the risk for malignant transformation, and within a few years, local relapses may therefore arise unexpectedly in these fields that stayed behind after surgery.[40, 44-52] Very recently, it has become possible to visualize premalignant fields using autofluorescence, which convincingly confirms their existence and dimensions.[53, 54] Although this is an advantage, still, the identification of

individuals with precancerous fields might considerably improve the early diagnosis of both primary and secondary HNSCC if suited protein biomarkers were available.
In summary, current major research questions in head and neck cancer research are related to accurate prediction of the malignant transformation of visible precursor lesions, the analysis of surgical margins to predict the risk for local relapse in treated HNSCC patients, the presence and staging of lymph node metastasis in the neck, and the prediction of distant metastases.

CANCER PROTEOMICS AND PROTEIN BIOMARKERS

Tumor biomarkers can be represented by DNA, mRNAs, proteins, but also by carbohydrates, or small molecules like metabolites and other cellular molecules.[55, 56] As biomarker could serve either a single molecule, a combination of multiple molecules, or a specific molecular profile. Ideally, tumor biomarkers should permit easy and reliable analyses, and the used assay should be cost-effective while showing high analytical sensitivity and specificity.[57] Notably, novel biomarkers should have added value beyond that of the current standards.[58]
Advanced genetic and genomic approaches for cancer biomarker discovery have recently been complemented by proteome analyses.[57] Proteins are important cellular molecules and more diverse than nucleic acids. Many regulatory processes occur post-transcriptionally, such as alternative RNA splicing, microRNA mediated transcript processing or translation regulation, protein activation, post-translational modification of proteins, or protein transport to the target organelle, which cannot be derived from the nucleic acid level. Proteins are also very attractive biomarkers, since they can be detected by ELISA in body fluids or by immunohistochemistry (IHC) in tissue, techniques that are easy, cost-effective and routinely applied in clinical pathology. The numerous proteomics tools that are currently available enable the study of a large variety of cellular protein characteristics like relative protein quantities and that of their post-translational modifications, protein cellular sub-localization, and the composition and stoichiometry of protein-complexes.

Sample selection: from model to cancer tissue

A protein biomarker indicative for carcinogenesis can be a protein that is differentially expressed in, or differentially secreted from tumor tissue compared to healthy control tissue. The ideal source of biomarkers are the proteins that are known to be causally involved in carcinogenesis. Besides these single biomarkers, also a specific change of multiple proteins leading to a specific protein expression profile, i.e. a ‘protein expression signature’, may

serve as indicator of carcinogenesis. As human tumor and control tissue is not always available in large enough quantities, often models are employed. Tumor cell lines are the easiest available source to identify potential protein biomarkers, in the sense that protein samples can be obtained in relatively large quantities for in-depth proteomics. Still, data should be interpreted with caution, since the detected protein levels may not always reflect the *in vivo* state.[59] Further, animal models may be utilized, in particular mouse models, as for most mouse proteins of interest human orthologs can be found. The advantage of using mouse models is that inbred strains can be used, whereby any heterogeneity due to genetic and environmental factors that play a role in studies with human samples can be circumvented. The approach that comes closest to studying human material is the use of xenograft models, immuno-compromised mice that are bearing orthotopic human tumors. Still, patient tissue, either as fresh frozen or as formalin-fixed paraffin embedded (FFPE) material, is the material that optimally reflects tumor physiology, but is unfortunately not always available in large enough quantities for in-depth proteome analyses. On the other hand, an estimated 20% of the proteins are secreted from the cell, thus cancers may secrete tumor-specific proteins, which can be detected in proximal fluids like saliva, the interstitial fluids adjacent to the tumor, or other body fluids such as blood and serum.

In the next sections the most current research on protein biomarker discovery in head and neck tumors is highlighted, propelled by the large improvements of proteomics technologies. Protein labeling methods like the use of three spectrally distinct fluorescent Cy-dyes or of distinctive mass-labels in the isobaric tagging (iTRAQ) reagents or other chemical labels have allowed multiplexing and the use of common standards, and have largely improved quantification reliability. Importantly, this permits analysis of tens of samples simultaneously, which is important for research on tissue and body fluid samples that are intrinsically heterogeneous, and that necessitate statistical analyses of the data sets. Moreover, developments have been made in relation to mass spectrometry, e.g. mass accuracy has enhanced, which resulted in improved confidence for protein identification, and also detection capacities have improved through shorter cycling times that allow increased throughput.

Tissue analysis

Protein biomarkers for head and neck cancer and the approaches for discovery thereof have been summarized in Table 1. We have limited the list to biomarkers that have been verified by other methods, ranging from Western blot to IHC or to functional analyses. It can be concluded from these data that the most widely used sample material is tissue, both as fresh frozen and as FFPE samples.[60-70]

Table 1. Overview of potential protein biomarkers for HNSCC

Sample	Approach	Biomarker [accession no.]	Verification	Biomarker for	Ref.
Tissue					
HNSCC (FFPE)	RPLC-MS/MS: MS-count of unique peptides per protein	desmoglein-3 [P32926]; cytokeratin 4 [P190013]; cytokeratin 16 [P08779]; desmoplakin [P15924]; vimentin [P08670]	IHC	Carcinogenesis	Patel (60)
HNSCC	IMAC30 protein chip arrays; SELDI-TOF MS	α-defensins 1-3 [DEF1-3]	Analysis using training and validation set; Tissue microarray; IHC	Tumor relapse	Roesch-Ely (61)
HNSCC	2D DIGE	Keratin 4 [P19013]; Keratin 13 [P13646]: Cornulin [Q9UBG3]; Small proline-rich protein 3 [Q9UBC9]	IHC; ROC; KMS	HNSCC premalignant tissue and Second Field tumor tissue	Schaaij-Visser (62)
OSCC	Cellular sub-fractionation (mitochondria/ cytosol); 2D GE colloidal Coomassie	14-3-3 sigma [P31947]	IHC	OSCC	Koehn (64)
HNSCC	iTRAQ/MDLC (strong cation exchange/ reversed phase)	14-3-3 sigma [P31947]; 14-3-3 zeta/delta [P63104]; S100-A7 [P31151]	IHC; WB; rt-PCR; Co-immuno precipitation assays; ROC; KMS	HNSCC	Ralhan (65); Matta (66)
Oral premalignant (leukoplakia)	iTRAQ	14-3-3 sigma [P31947]; 14-3-3 zeta/delta [P63104]; hnRNPK [P61978]; S100-A7 [P31151]; PTHA [P06454]	IHC; WB; rt-PCR; ROC	Epithelial dysplasia (leukoplakia)	Ralhan (67)
OSCC; OSCC cell lines	$^{16}O/^{18}O$-labeling; 2DLC	Ubiquitin cross-reactive protein [P05161]; Serpin H1 [P50454]; Beta ig-h3 [Q15582]; Signal transducer and activator of transcription 1-alpha/beta [P42224]; Thymidine phosphorylase [p19971]; Filamin-A [P21333]; Filamin-B [O75369]; Superoxide dismutase [Mn], mitochondrial [P01479]; Fascin [Q16658]; GTP-binding protein 1 [P32455]; Annexin A3 [P12429]; Carbonic anhydrase 2 [P00918]	WB; IHC	Tissue markers for OSCC tumors	Chi (68)
OSCC	2D GE; Coomassie	Rack1 [P63244]	WB; IHC; RT-PCR; Rack1 si-RNA to assay OSCC proliferation and apoptosis	Severity dysplasia	Wang (69)
OSCC (FFPE)	LC-MS; Label-free quantitation	Keratin 13 [gi62897663]; Keratin 4 [109225249]; Transglutaminase 3 [gi80478896]; Annexin I [gi442631]	WB; IHC; rtPCR; cytosine methylation analysis	OSCC (Epigenetically silenced gene)	Negishi (70)

Sample	Approach	Biomarker [accession no.]	Verification	Biomarker for	Ref.
Tissue secreted proteome: serum/saliva/secretome					
Orthotopic xenograft mouse model OSCC/ serum analysis	DIGE & iTRAQ/2DLC	EGFR [A8K2T7]	ELISA; WB; IHC	OSCC tissue and tumor invasion	Bijian (85)
HNSCC serum	Magnetic chemical affinity beads (IMAC-copper)	MALDI-TOF peak pattern profiles	none	HNSCC (serum profile)	Freed (84)
OSCC saliva	Strong cation exchange/ reversed phase LC; 2D GE	S90K/Mac-2 binding protein; S100-A9; CD59; Profilin; Catalase	ELISA; WB; ROC	OSCC	Hu (87)
HNSCC saliva	2D DIGE	S100-A9	WB	Early detection	Dowling (88)
OSCC cells in saliva	Peptide Free Flow Electro-phoresis; SCX	Signal transducer and activator of transcription 3 [P40763]; Thioredoxin-dependent peroxide reductase, mitochondrial [P30048]; Serpin B3 [P29508]	WB	OSCC	Xie (89)
OSCC cell line secretome	1D SDS-PAGE	Mac-2 binding protein	WB; IHC; ELISA on serum; ROC; Mac-2 BP siRNA and assay cell proliferation, invasion, migration	OSCC	Weng (90)
Autoantibodies					
Against ESCC antigens	2D-WB of ESCC cell line	Serum antigens: Heat shock 70 kDa protein 9B; Stress-70 protein, mitochondrial	ELISA (serum levels); IHC	ESCC	Fujita (92)
Against OSCC antigens	2D-WB of OSCC and fibroblast cell lines	Serum antigen: Sideroflexin-3 [56462561]	ELISA (serum levels); IHC; ROC	Reflects tumor burden: correlation with primary tumor size/recurrent tumor/pre- *vs* post-therapy	Murase (91)
Cell models					
OSCC	2D GE; Silver stain	Annexin A1 [gi113944]; Annexin A2 [IPI00455315.3]; Cathepsin B [IPI00295741.4]; S100-A6 [IPI00027463.1]; Keratin 17 [IPI00450768.5]	WB; IHC	OSCC	Zhong (95)
HNSCC p53 pathway disruption	2D DIGE	Peroxiredoxin-1 [Q06830]	IHC on HNSCC tissue	P53 pathway disruption in HNSCC	Schaaij-Visser (94)
OSCC	2D DIGE	Lin -7C	IHC; qRT-PCR; Lin-7C functional assays; invasion assay; in vivo metastasis assay	Invasive capacity OSCC cells/local metastasis in tumor	Onda (93)

Note: Accession numbers are given as indicated in the publications; IHC=immunohistochemistry; ROC= Receiver operator characteristics analysis; KMS=Kaplan-Meier survival analysis; WB=Western blotting

Tumor tissue is apparently considered the most reliable source of material to find novel biomarkers, although the amount of sample proteins that can be obtained is limited. Epithelial tissue is quite heterogeneous, thus laser capture microdissection or manual microdissection has been applied to obtain 'pure' squamous tissue cells, for which a few hundred thousand up to a few million cells could be collected.[60, 62, 68, 70] In most of these studies tumor versus premalignant and/or normal tissue is analyzed, but the classification that has been performed is usually based on histopathology using a hematoxylin- and eosin-stained section of each investigated tissue block. Although histology is considered 'the gold standard', it is not always conclusive. It has been well established that head and neck carcinogenesis is driven by genetic alterations resulting in p53 pathway disruption[38-40] and associated with allelic losses at chromosome arms 3p, 9p and 17p[41-43]. It may therefore be preferable that in addition to histopathological grading, genetic analyses for allelic losses are performed to confirm at least the state of the premalignant or normal tissue.[62] A less labor-intensive approach is immunostaining for mutated p53, but this is not always conclusive, since not all HNSCC tumors or premalignant tissues contain p53 missense mutations. Only for p53 missense mutations the changes can be detected immunohistochemically. Analysis of human tissue has the disadvantage that the biological noise is increased due to genetic variation between subjects when compared to mouse- or cell models. However, biological noise can be decreased and statistical power enhanced when paired human samples are employed (e.g. normal and tumor tissue of the same patient).[62, 64, 65, 70]

A rather novel and very promising approach in HNSCC research is the utilization of FFPE tissue. [60, 70] The advantage of using FFPE material for discovery-driven biomarker research is that enormous archives of patient samples exist (in contrast with biobanks for fresh-frozen tissue), and also, that corresponding pathological and clinical patient records are present, with known clinical outcome, which permits retrospective analysis for e.g. disease outcome.

On the other hand, FFPE tissue samples are fixed in a buffered formalin solution, resulting in cross-linking of intracellular proteins to other biomolecules, which is highly beneficial for preserving the morphological details within the cells. However, this has adverse effects for proteome analysis with mass spectrometry, as extracted peptides are modified at multiple sites by formaldehyde or exist as cross-linked peptides, which hampers protein identification. Nevertheless, it is assumed that proteins are protected to a certain extent from these modifications due to their tertiary and quaternary structures, and that sufficient unmodified peptides can be derived from FFPE material. Hood *et al.*[71] for example, have performed protein/peptide analysis for comparable tissue sections of mouse liver that were either fresh frozen or FFPE-prepared, and analyses of both samples by mass spectrometry provided similar results according to the number of peptides detected and proteins identified.

Identification of candidate protein biomarkers is one issue, but clinical validation seems the major obstacle at present. In most studies some verification or validation is presented, but usually in retrospective designs and small selected sample sets. Follow-up research in multicenter prospective screening studies and large clinical trials, needed to allow future implementation of these biomarkers for routine clinical diagnosis, has not been performed yet.[57]

The analysis of HNSCC tissue specimen has not always provided identical biomarker candidates, which may be explained by differences in study design. Tissue samples were for example microdissected[60, 62, 68, 70, 72, 73], or not microdissected[61, 64, 65, 67, 69] and instead divided into mitochondrial and cytosol fractions[64]. Obviously, it can be expected that other protein biomarkers are identified when the investigated tissue contains large amounts of stroma besides squamous cells. Also the applied analytical approaches differ widely. Proteins from tissue lysates were separated through 2D gel electrophoresis (2D GE) or 2D liquid chromatography (2D LC) (Table 1), approaches that are known to provide complementary results due to differences in selection of proteins with extreme iso-electric points, overall size, or hydrophobicity. Even though, five proteins were detected and verified in more than one independent study. Common markers are the proteins 14-3-3 sigma[64, 65, 67], 14-3-3 zeta/delta[65, 67], and S100-A7[65, 67], all upregulated in HNSCC, while keratin 4[60, 62, 70] and keratin 13[62, 70] were downregulated in HNSCC. The 14-3-3 sigma and 14-3-3 zeta/delta proteins recognize phosphorylated serine/threonine-containing motifs, and are involved in many signaling pathways, including cell cycle regulation. The 14-3-3 proteins promote cell survival through interaction with for example EGFR, Bcl-2 and cdc25. These proteins have a potential role in carcinogenesis, although association with loss of expression of both 14-3-3 proteins has been described.[74, 75] Follow-up proteomics studies using co-immunoprecipitation have been performed that showed binding of 14-3-3 sigma to 14-3-3 zeta/delta and to NFκB, β-catenin, and Bcl-2 in oral cancer cells (HSC2 cell line) and not in negative controls.[66] S100-A7 is a calcium-binding protein of the S100 protein family and was found to be involved in dysregulation of keratinocyte differentiation, and may also be a marker for invasion.[76] The observed increase in S100-A7 expression may be explained by increased hypoxia and reactive oxygen species, that both occur in HNSCC, and has been described earlier as a marker for breast cancer progression.[77]

Keratins 4 and 13 are a well-known keratin couple, present in the suprabasal layer of mucosal stratified squamous epithelial cells[78], and form the intermediate filaments of the cytoskeleton in these cells. As for all keratin couples, the proteins form a heterotetramer, containing two neutral type II keratins (keratin 4) and two acidic type I keratins (keratin 13). Keratin 4 and 13 are best known for their role in the hereditary disorder ‘white sponge

nevus', which can be caused by mutations in either one of these keratins.[79] This disease causes benign white lesions in the oral cavity that are easily mistaken for leukoplakias, which are known precancerous changes. Hence, the change in keratin expression might well explain that some precancerous fields present as visible lesions. Since keratins are involved in epithelial cell differentiation, they are often found to be downregulated in epithelial tumors, and more specifically, in head and neck tumors.[60, 80-82]

For some of the potential biomarkers it has been found through receiver operator characteristics (ROC) analysis, Kaplan-Meier survival analysis and/or Cox-proportional-hazards-models that a combination of two or more of the verified biomarkers showed even stronger statistical power. For example Ralhan *et al.* have shown that for discrimination between oral premalignant lesions and histological normal oral tissues the combination of increased 14-3-3 sigma 14-3-3 zeta/delta and S100-A7 levels demonstrates increased sensitivity and specificity according to both the iTRAQ-scores and the IHC-scores.[65] In a follow-up study Matta *et al.* have demonstrated that patients with tumors showing normal expression of both 14-3-3 sigma and 14-3-3 zeta/delta have a significantly increased disease-free survival (with a median of 38 months) compared to patients whose tumors showed enhanced expression of these 14-3-3 proteins (with a median of 13 months). [65, 66]

Schaaij-Visser *et al.*[62] recently described a panel of biomarkers that could be exploited to predict local relapse in surgically treated patients by immunostaining of the surgical margins. They have used paired samples of which the normal or (pre)malignant state (normal, precancerous, tumor) was performed with genetic analyses, and could verify four protein biomarkers, keratin 4, keratin 13, cornulin and small proline-rich protein 3. These proteins showed strong reduction of expression in tumor surgical margins, also on independent sample sets.[62] In a retrospective case-control study it has been shown that for two of these protein biomarkers, keratin 4 and cornulin, the loss of expression in surgical margins of the resected tumor specimen proved to be significantly associated with development of local relapse. At the same time they could conclude that this approach outperformed histological grading, which is the current 'gold standard' method.[62] These proteins, keratin 4 and cornulin, are, as described above, markers of cellular differentiation, which may at first sight disqualify these protein as suited HNSCC biomarkers, however, the results have demonstrated that their reduced expression can be very well utilized in a retrospective study, and this study has also shown the added value of proteome research for biomarker discovery.

Analysis of tumor-secreted proteins in body fluids

It has been estimated that approximately 20% of the cellular proteins are secreted. This would allow identification of novel HNSCC biomarkers by tissue 'secretome' analysis that can be detected in saliva as tumor proximal fluid, or serum. Serum can be relatively easy sampled in large quantities, which makes it a promising sample to discover suitable protein biomarkers. Biomarker discovery in serum is however not straightforward, since the generally diluted concentrations of potential tumor markers require special care with respect to sample preparation, necessitate reproducible depletion of the 12 to 20 most abundant proteins, and require a study design that avoids introduction of confounding factors.[83] Such serum analysis has recently been described using MALDI-TOF analysis[84], which revealed peak pattern profiles specific for HNSCC. However, potential biomarker proteins could not be identified using this approach. In contrast, 15 specific tumor proteins could be identified from mouse serum using a mouse orthotopic xenograft model for oral squamous cell carcinoma (OSCC).[85] Cancer tissue from patients with defined pathology and distinct invasive property was transplanted into the tongue of RAG-2γ (c) mice, and the same pathologic, tumorigenic and invasive potential was detected for these mice as earlier for the patients. Serum proteomics revealed 15 proteins that were differentially expressed in cancer bearing mice compared to control. One of these was the EGFR protein, which seems a promising candidate as this is a known tumor marker for HNSCC. EGFR is reported to be overexpressed in approximately 80% of the HNSCC tumors, thereby stimulating a number of downstream signaling events correlated with increased tumor size, decreased radiation sensitivity, and increased risk of recurrence.[86] However, subsequent validating studies still have to be awaited.

Besides serum, also other body fluids can be used to identify or screen protein biomarkers, and for HNSCC saliva seems an ideal source. Human saliva is easily collected by a non-invasive method, and relatively large quantities can be obtained. Saliva proteome analyses were performed using OSCC saliva as source.[87, 88] These studies provided the S100-A9 protein as common potential biomarker. Since saliva also contains many cells that are mainly exfoliated from the oral mucosa, also this cellular source was investigated for potential biomarkers.[89] This also allowed detection of over 30 bacterial strains from the saliva, of which a few are known as being associated with the presence of cancer.

Secretome analyses have also been performed using relevant HNSCC cell lines derived from the oral cavity[90], but not yet on tumor interstitial fluid. Interestingly, this latter study and the earlier mentioned saliva proteome analysis[87], have both identified Mac-2 binding protein (Mac-2 BP) as potential biomarker. This potential serum- and saliva biomarker protein has been further verified by patient serum ELISA analysis. The potential role of this protein in

carcinogenesis has been assessed by knockdown of Mac-2 BP in OEC-M1 cells using siRNA, followed by analysis of cellular proliferation, invasion and migration, to establish if this resulted in known cancer-associated phenotypes.[90] At days 3 to 6 post-seeding, a drastic growth retardation was detected for the Mac-2 BP-silenced cells compared to the control, while the observed lower proliferation rate by flow cytometry suggested that cells were arrested in the G2/M phase. Transwell migration and invasion assays showed severe impaired capacities of migration (60-70% reduction) and invasion (50-90% reduction). These results indicated that Mac-2 BP might be causatively associated with tumorigenesis and makes it a very potential biomarker for serum[90], and maybe also for saliva[87]. Although the exact function of Mac-2 BP in tumorigenesis is still not known, the protein is known to enhance cell-cell and cell-extracellular matrix adhesion. The elevated expression levels of this protein have also been detected in tissue and sera of patients with various other cancer types (breast cancer, non-/Hodgkin's lymphoma, ovarian cancer, lung cancer, colon cancer and nasopharyngeal cancer)[90], which merely qualifies this protein as generic cancer biomarker.

Autoantibodies as tumor biomarkers

Secreted tumor proteins may induce formation of autoantibodies, which can be detected in patient serum. The approach for these studies is to separate protein lysates from human squamous cell carcinoma cell lines by 2D GE, followed by Western blotting using patient sera.[91, 92] Recognized antigens have been identified by mass spectrometry. This research delivered heat shock protein 70 as early marker for SCC of the esophagus[92], and sideroflexin 3 for OSCC[91]. It has been suggested that these autoantibodies might be used to establish effective new immunotherapies, besides their employment for early diagnosis of these tumors.

Cell models for HNSCC

Tissue cultures of HNSCC cell lines provide ample proteins for in-depth proteomics analyses, and have therefore been widely explored to detect novel biomarkers. Onda *et al.*[93] investigated OSCC-derived cell lines and detected downregulation of Lin-7C compared to normal cells. Verification by IHC on 80 OSCC tissue samples with a number of different pathologies, they detected that the Lin-7 protein might serve as a novel biomarker for early detection of OSCC metastasis. Namely, most patients with primary OSCC showing Lin-7C downregulation had regional lymph node metastasis, while tumors of patients without metastatic lesions were still positive for Lin-7C.

Other groups used *in vitro* cell models for HNSCC biomarker studies.[94, 95] For instance to investigate OSCC *in vitro*, Zhong *et al.*[95] used several cell lines that reflect different stages of carcinogenesis. They used a human oral epithelial cell line that was transfected with the HPV16 E6/E7 genes (HIOEC), a cancerous cell line (induced by benzo(a)pyrene; HB96), and a cell line that reflected the early stage of carcinogenesis (HB56). They found five proteins that correlated with the pathological differentiation grade of cancerous tissues, i.e. annexins A1/A2, cathepsin B, S100-A6 and keratin 17. As described earlier in this review, it is known that mutation of the p53 gene occurs in 50% of all cancers and this percentage is even higher in head and neck tumors.[96, 97] In HNSCC, the p53 pathway can be abrogated by mutation of the p53 gene, targeted degradation of the p53 protein by the HPV E6 protein, and inactivation of any of the modulators of p53.[96] Different types of p53 mutations have been found with distinct effects on expression of the mutated p53 protein, as p53 can either be degraded or upregulated, which hampers straightforward application of p53 as a protein biomarker. Schaaij-Visser *et al.*[94] identified many differential proteins related to p53 dysfunction by using a conditionally immortalized oral squamous cell model for these three common ways of p53 inactivation. These models mimicked either p53-missense or p53-nonsense mutations, and a third model was used that imitated p53 inactivation through the HPV E6 protein. This study revealed a few interesting proteins that are indicative of p53 inactivation, and of these, the peroxiredoxin-1 protein also allowed immunohistochemical discrimination between normal epithelium and precursor field tissue with a *TP53* mutation.

Dysregulated protein networks

To decipher how the proteins that were identified in the proteomics screens might be related to HNSCC and/or the processes that underlie carcinogenesis, gene ontology and/or network analyses have been performed.[67, 68, 94, 95] Chi *et al.* have detected 53 upregulated and 27 downregulated proteins associated with HNSCC, and gene ontology analysis has revealed the involvement of the interferon signaling pathway, as 10 of the upregulated proteins were members of the interferon-stimulated gene family.[68] Schaaij-Visser *et al.* have performed gene ontology analysis on the set of differentially expressed proteins in response to p53 abrogation, which indicated that the p53 protein plays an important role in glucose metabolism. A change in glucose metabolic processes associated with cancer is well-known as the Warburg effect.[94] Using a conditionally immortalized oral keratinocyte cell model Schaaij-Visser *et al.* demonstrated that this Warburg effect might be related to p53 inactivation, an intriguing novel observation. Further, pathway analyses with ingenuity pathway analysis software have been exploited, i.e. software that links the experimental data to earlier data found in literature, which associated experimental data in a leukoplakia

study to inflammation[67], and also related participation of the p53 and Myc-pathways to OSCC carcinogenesis[64].

FUTURE PROSPECTS

SRM as novel approach in the clinical validation process

Clinical validation is the most important and probably also the most time-consuming phase of cancer biomarker discovery, particularly to perform the large prospective clinical studies required to prove the clinical value of candidate biomarkers necessary before implementation is considered. In most studies Western blotting, ELISA and/or IHC have been used to validate detected differential protein expression, not only in the sample set used for their discovery, but importantly, also in independent sample sets. Such experiments can provide valuable information on which proteins can be used for further clinical validation. However, suitable antibodies for Western blotting, ELISA, or IHC are not always available, and it is excessively expensive and time-consuming to develop these antibodies for all potential protein biomarkers. Recently, selected reaction monitoring (SRM) has been introduced as a mass spectrometry based method that can be exploited to specifically select and quantify promising protein biomarkers in serum or tissue.[98] In the future, this technique may complement or possibly replace Western blotting for biomarker verification and for selection of potential biomarker candidates.[99] SRM-analyses may thus help to determine which antibodies should be developed for clinical use via ELISA or IHC. In addition, SRM might even be applied to monitor differential expression of tumor proteins in blood and saliva. Initial SRM studies have been reported for prostate and breast cancer[100, 101]; however, no SRM studies have been described for HNSCC yet.

Shifts in sample choice and depth of proteome research

As can be concluded from Table 1, most recent head and neck tumor proteomics studies make use of fresh frozen tissue material which was obtained from patients through surgery, as this material reflects tissue physiology probably best. The vast amount of FFPE tumors in pathology archives, however, makes it worthwhile to further improve recovery of proteins or peptides from FFPE material. This will certainly enhance the verification and clinical validation process.

Notwithstanding the above described promising outlook of this field, there are also concerns. As indicated many very promising biomarker proteins for HNSCC have been detected, but some of the potential biomarkers that are listed in Table 1 have also been detected in many other biological and biomedical research projects (reviewed by Petrak *et al.*[102], and Wang *et*

al.[103]). These are proteins from families such as the heterogeneous nuclear ribonucleoproteins, the annexin family, the peroxiredoxins, the 14-3-3 family, the cathepsins, the serpin family, and the superoxide dismutase family. Differential detection of proteins from these families is unfortunately not specific for HNSCC or cancer in general, but has also been found in many studies with other experimental objectives. Petrak *et al.* and Wang *et al.* suggest that the frequent detection of these proteins may be linked to the used proteomics platform (2D GE), which is indeed true for some of the research projects listed in Table 1[64, 94, 95], however also many other platforms deliver these commonly detected proteins as potential biomarkers[60, 67, 68, 70, 89]. Most likely another important factor for common detection of proteins in a wide diversity of comparative studies is that for all these proteomics projects only the few hundred most abundant proteins have been considered. For the HNSCC tissue analyses, this is probably due to the fact that the amount of material that can be obtained is typically limited. Even when for example cellular sub-fractionation has been performed[64], or free flow electrophoresis followed by SCX[89], or by MUDPIT-like approaches[65], a few of the commonly detected proteins were found (Table 1). For serum and saliva, the sample quantities as such are not the limiting factor, but the very wide dynamic range of protein expression levels, and despite efficient depletion methodology for the 12 to 20 most abundant proteins, the remaining dynamic range is apparently still larger than can be covered by the current proteomics methodologies. Thus far, we are not able to perform a full, in-depth proteome analysis to identify many more differential proteins, like a low-abundant transcription factor as p53, that are causally related to the cancer processes. Therefore the ever ongoing developments in proteome technologies should also be focused on expansion of techniques that permit progressively in-depth tissue and serum/saliva proteome analyses, such that many more low-abundant proteins can be reliably quantified. Proteins from known and yet unknown networks involved in HNSCC could then be discovered.

Changes in HNSCC therapy and consequences for protein biomarker discovery

As indicated above, the major research questions in head and neck cancer research have thus far been related to the accurate prediction of malignant progression of visible precursor lesions, the analysis of surgical margins to predict the risk for local relapse in treated HNSCC patients, the accurate staging of the clinically N0 neck, and the prediction of distant metastases. However, the research focus needs to be rapidly adapted. For patients with advanced stage tumors (stage III/IV; about 60% of the patients) the treatment of surgery with postoperative radiotherapy has more and more been replaced by chemo-radiation for several sites, i.e. concurrent treatment with cisplatin and radiation. However, only 50% of the

patients responds well to chemo-radiation, and these patients should better have been treated by surgery and postoperative radiotherapy, which creates a problem for tailoring treatment to the individual patient.[104, 105] Biomarkers that allow prediction of treatment efficacy become therefore increasingly important. In addition, the availability of tissue specimens will be reduced due to this change in treatment, and as a result, future proteome research for HNSCC may shift focus to body fluids such as saliva, and serum.

An upcoming treatment modality for HNSCC is the targeted therapy using biological agents. Dysregulation of signaling cascades in head and neck carcinogenesis involves pathways like that of EGFR.[106] This information is increasingly being applied for targeted therapies for HNSCC using inhibitors, i.e. monoclonal antibodies like cetuximab, which binds to the extracellular domain of EGFR and inhibit the signaling cascades that drive tumor growth. Phase I/II and III studies using these agents alone or combined with the more classical treatments are currently in progress. However, these trials often show limited efficacies[107], and future protein biomarker discovery for HNSCC may therefore also be important to monitor the effects of current and novel therapeutic strategies.

Finally, it has been shown that HNSCC in fact consists of three independent tumor classes that show a different prognosis and might benefit from specific targeted treatments. First, there are tumors caused by HPV infection, that can readily be distinguished by a p16 immunostaining in combination with PCR on HPV DNA.[108] However within the HPV-negative tumors two groups have been distinguished on basis of their genetic profile.[108] One group showed major numerical DNA changes and was assigned as high-CIN (i.e. chromosomal instability). The second group hardly showed any genetic changes and was assigned as low-CIN. These groups seemed to have both a different etiology and a different prognosis, therefore protein biomarkers to distinguish both HPV-negative groups might be a great aid, not only for accurate diagnosis and classification, but also to identify the specific pathways driving these tumors.

CONCLUSIONS

Many protein biomarkers have recently been detected from HNSCC tissue, serum, and saliva samples, but also from appropriate models for HNSCC. Although some of these biomarkers have been found in a wide diversity of other studies, often not even related to cancer, many other proteins have been detected that show high potential to be further developed for clinical applications for HNSCC. Verification of these potential biomarkers has been performed using immunotechniques (ELISA, Western blotting, IHC). On the other hand, SRM as mass spectrometry technique has been reported as upcoming approach that might support or eventually replace antibody-based methods. Future proteomics methodology development should focus on improved methods for in-depth analyses, to permit detection of many more low-abundant biomarkers that explain the underlying processes related to HNSCC.

Also, it seems inevitable that focus in HNSCC will shift from tissue analysis to analysis of serum and saliva due to changes in therapy. Finally, it will become increasingly important to investigate HNSCC treatment efficacy and to improve the tailoring of targeted therapies by serum and saliva analyses.

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Chapter 7

Summary, discussion and future perspectives

SUMMARY

Despite the development of better treatment options[1, 2], the prognosis of head and neck squamous cell carcinomas (HNSCC) has only moderately improved during the last decades.[3] Major causes for this are: the high number of patients that present with advanced stage of disease resulting in frequent locoregional relapses despite extensive treatment by combined modalities; the high frequency of second primary tumors that are difficult to treat, particularly when diagnosed at a late stage; and the lack of an effective systemic treatment to prevent distant metastases. This emphasizes the need for early diagnosis of both primary and secondary tumors, and the development of novel treatment approaches. HNSCC carcinogenesis follows more or less distinct phases over an estimated 15 to 20 years, and is driven by the accumulation of genetic alterations.[4, 5] Head and neck tumors arise in a field of premalignant cells that already harbors early genetic changes, i.e. a precursor or precancerous field.[4, 6] Early detection of these fields, in particular those with a high risk of progression, might allow the increase of surveillance and application of novel preventive interventions. Dysplasia grading of the surgical margins is of limited use in individual cases. Genetic analyses could be used to identify precursor fields and predict progression, but these analyses are laborious and cannot be easily implemented in the daily diagnostic practice.

Proteomics is the field of research that aims to study the proteome, i.e. all proteins present in a cell or tissue at a certain time point.[7] Proteomics approaches in cancer research enable the identification of protein biomarkers, that is those proteins, present in tissue or body fluids, that are indicative of the presence, state or behavior of a tumor or precancerous lesion.[8, 9] The development of protein biomarkers is characterized by different phases, starting with an initial discovery of the possible protein candidates, to the final thorough clinical validation of these candidates in prospective multicenter trials. The studies described in this thesis report the discovery and initial clinical validation of protein biomarkers that can predict malignant progression of HNSCC precursor fields.

One of the earliest events in HNSCC carcinogenesis and a characteristic of the majority of precursor fields, is disruption of the p53 signaling pathway.[10, 11] The p53 pathway maintains the integrity of the genome and induces apoptosis in response to DNA damage, and inactivation of the *TP53* gene results in uncontrolled cellular proliferation.[12] For the study described in **Chapter 2**, we used a conditionally immortalized squamous cell model in which p53 pathway disruption was mimicked. By comparing the proteome of this cell model with that of normal keratinocytes, a list of potential protein biomarkers for p53 pathway disruption was discovered. For six of these proteins the differential expression was validated by

immunostaining, and one protein, peroxiredoxin-1, could discriminate between normal tissue and precursor tissue.

In Chapter 2 we noted that the extrapolation of the identified biomarkers in cell models to clinical applications in tissue samples, was not straightforward, and therefore, we switched to biomarker discovery using tissue biopsies. For the research described in **Chapter 3**, we selected and genetically characterized normal, precursor and tumor tissue from eight patients. The proteomes of these different tissue types were compared to identify proteins with a different expression level. The differential expression of the most promising protein biomarkers was confirmed by immunohistochemistry. We demonstrated that expression of four proteins, keratin 4, keratin 13, cornulin and small proline-rich protein 3, was not only tremendously decreased in tumors, as compared to normal mucosa, but also in an independent series of severe dysplasias, mucosal tissue at a high risk of malignant progression. This suggested that we found promising candidates that deserved further clinical validation, and we decided to investigate whether these proteins might predict malignant progression of precancerous tissue.

Leukoplakias are white lesions in the mouth that cannot be classified as any other disease and carry a risk of malignant progression, estimated at 2 to 3% per year.[13] At present the risk assessment of leukoplakias is based on histological grading, but this is somewhat subjective and does often not allow prediction of malignant progression in individual cases.[14] In **Chapter 4** we describe the evaluation of keratin 4, keratin 13 and cornulin expression in leukoplakia lesions for the prediction of malignant transformation. Forty-eight leukoplakias, of which 36 had not progressed and 12 progressed to HNSCC, were immunostained for these three proteins. Kaplan-Meier survival analysis revealed that for this type of lesions keratin 4, keratin 13 or cornulin expression showed no association with progression. The most likely reason for this result was that the proteins tested are proteins involved in cellular differentiation, which is known to be disturbed in leukoplakia lesions that frequently show hyperkeratosis. Dysplasia grading seemed to be more successful to predict malignant progression of leukoplakia lesions.

In patients treated by surgery, the tumor is excised, but often parts of the precursor field are left behind, as these are usually not macroscopically visible, and might cause local relapse.[15-19] In **Chapter 5** we investigated whether expression of keratin 4 and cornulin in the surgical margins could predict local relapse in patients surgically treated for HNSCC. The resection margins of 23 patients who remained disease-free and 23 patients who developed a local relapse were immunostained for cornulin and keratin 4. Kaplan-Meier survival analysis demonstrated a significant association between decreased keratin 4 and cornulin expression and local relapse, and a very strong association when both protein

markers were combined. Presence of dysplasia was not associated with local relapse. This was a surprisingly positive result of our studies that requires further validation.

During the last few years the number of proteomics studies involving HNSCC has grown exponentially. Especially new sample types and selections are presented as well as initial large-scale protein biomarker validation studies including survival analysis and clinical applications. Besides clinical validation, several reports describe functional validation by RNA interference, migration and invasion assays. In **Chapter 6** an overview of the studies on HNSCC proteomics published in the last years is provided and future recommendations for this field of research are given.

DISCUSSION AND FUTURE PERSPECTIVES

HNSCC proteomics

A great challenge for clinical proteome-wide approaches is the choice and preparation of protein samples that need to be compatible with either gel-based, chromatography-based and/or mass spectrometry-based separation, identification and quantification methods. Cell line samples are relatively easy to handle and large quantities can be obtained, however, the results need to be interpreted with caution and cannot always be directly applied on actual patient samples, as we experienced. Still, cell lines will remain important for functional in-depth validation studies to investigate the biological mechanisms behind the identified protein biomarkers.

Fresh samples from either tissue or body fluids, are more difficult to obtain, and in general the amounts of available protein become limiting, which hinders proteome analysis and limits the number of analyses to be carried out. In addition, tissue samples are always mixtures of cell types and require preparation steps for enrichment or isolation of specific cell populations.

In our HNSCC biomarker discovery studies we observed that analysis of tissue samples is preferable. To improve the reliability of the findings, proper selection, characterization and handling of the samples is important. This is why we chose to genetically characterize the patient tissue samples to ensure that normal and precursor tissue was selected, and microdissected the epithelium to rule out contamination with underlying stroma or other tissue. Further, paired statistics allowed us to find highly significant consistent differences even with a relatively low number of patients.

Huge resources of clinically characterized HNSCC tissues with patient follow-up data could become available if protocols for extraction of proteins from formalin-fixed paraffin-embedded (FFPE) tissues would be optimized further.[20, 21] Even though the use of formalin-

fixed tissues for proteomics is still in its infancy, optimization of extraction protocols would be worthwhile.[22] Other promising sources for HNSCC protein biomarkers that could be investigated, are plasma, serum, tumor secretomes and, in particular for oral and oropharyngeal cancer, saliva.[23-25]

For the separation and quantification of proteins we chose to use two-dimensional differential gel electrophoresis (2D-DIGE) as it is still the most reliable method that allows simultaneous analysis of over 20 different samples. A limitation of the 2D-DIGE technology, when applied without further fractionation, is that it mainly focuses on the abundant proteins, which is actually only a fraction of the whole proteome. This explains why we did not find known oncoproteins or proteins involved in signaling pathways. Also, with this method all proteins are separated and quantified, but often only a subset of proteins that meet the criteria for differential expression are identified and those with relatively small fold changes or less stringent p-values were disregarded. This filtered view on the proteome limits gene ontology and pathway analyses. Subcellular fractionation or functional fractionation combined with 2D-DIGE would probably allow more in-depth proteome analysis. Also other label-based methods, such as iTRAQ[26], that enable quantification and identification of all proteins, have been developed and optimized for multiple samples in the last years. Label-free shotgun proteomics is a very straightforward option and, although it requires a stable mass spectrometer, is a good option for biomarker discovery.[27]

Probably the most time-consuming and also the most neglected phase of HNSCC biomarker development is the clinical validation. However, during the recent years the emphasis has started to move from only discovery of possible candidates towards initial clinical validation as also described in Chapter 6. Difficulties in the clinical validation phase are the selection of the most promising markers, availability of well-documented specimens with known outcome, and the availability of tools, usually antibodies. Gene ontology, network and pathway analysis tools are now available that can facilitate the selection of potentially successful protein biomarkers and provide insight in the biological mechanisms involved. Recently, selected reaction monitoring (SRM) was introduced as a mass spectrometry based method that can be used to specifically select and quantify previously identified proteins in a complex protein mixture [28]. In the future, this technique could be used to verify differential expression and select potential biomarker candidates. Further, this might also be an ideal tool to monitor expression of preselected peptides or proteins in plasma, saliva and secretomes.

Clinical validation is very much depending on the selected patient cohort and this emphasizes the importance of formulating a clear clinical hypothesis beforehand. However, even when the clinical validation seems straightforward starting with a well-defined clinical

hypothesis, surprises will occur. In our studies, we found that keratin 4 and cornulin could predict the development of local relapse in the surgical margins of treated HNSCC patients, but not the malignant progression of leukoplakias. For dysplasia grading, the current gold standard for both applications, the opposite was true.

These retrospective studies would have been impossible without the proper collection of patient materials and management of follow-up data. The initiation and continuation of these so-called biobanks will start to play a bigger role in the future of clinical proteomics and biomarker discovery. Besides the collection of freshly frozen patient tissues, also easy accessible materials such as serum plasma, saliva and brushed cells might be collected and stored for later validation experiments.

Future applications

Large scale, prospective, multi-center validation studies will be necessary to further unravel the value of keratin 4 and cornulin as HNSCC biomarkers for prediction of local relapse. The application of this approach will change however. The last few years the treatment of HNSCC is changing to radiation or chemoradiation instead of surgery combined with radiation. Only tumors of the oral cavity are and will be treated by surgery, and future studies on surgical margins should therefore focus on these tumors. The described data in this thesis can be used to set a cut-off score for the level of expression in the surgical margins that corresponds to the desired specificity and sensitivity to predict development of local relapse. A prospective multicenter study should focus on a consecutive series of patients with oral cavity tumors. Inclusion criteria might be squamous cell carcinoma, tumor-free surgical margins and N stage <N3. These inclusion criteria would ensure that relatively few patients would be lost due to follow-up by regional recurrences or distant metastases, and reduce the number of local recurrences due to residual cancer, that might have a negative effect on the statistical associations. A consecutive series would also allow a more reliable analysis of all prognostic factors predicting local relapse such as the lymph node status in the neck, and analyze associations of these parameters with immunostaining of the surgical margins. If other methods, such as allelic loss analysis or p53 immunostaining, can be included the results can be compared and possibly new associations between the various parameters might be identified.

A potential caveat in such a trial is the recent discovery of the existence of several genetically defined classes of HNSCC.[29] HNSCC should be divided in HPV-positive and HPV-negative tumors, that are different with respect to genetic changes[30], expression profiles[31] and prognosis[32, 33]. In addition, it was very recently shown that the HPV-negative tumors should also be classified in at least two groups, one with many genetic changes

(high-CIN) and one with hardly any genetic change (low-CIN), a division that also seemed of prognostic relevance. HPV might be a lesser problem in the planning of a subsequent study as the frequency in oral cavity tumors is relatively low.[30] The relevance of the other classification in low-CIN and high-CIN is less clear. A simple assay to detect the presence or absence of high-CIN and low-CIN would be of help to evaluate whether this parameter might be confounding for local relapse.

Besides screening the surgical margins of treated patients, other applications can be considered. The first recent successes in visualizing HNSCC precursor fields by autofluorescence might turn out to be a great aid to select the mucosal regions at risk of malignant progression.[34, 35] Hence, these visualization tools in combination with biopsy and keratin 4/cornulin immunostaining might be a suitable approach for the monitoring of patients who are scheduled for treatment regimens that do not encompass surgery. In these cases resection margins are not available. In addition, immunostaining for protein biomarkers such as cornulin and keratin 4 might be of use for non-invasive screening for HNSCC or precursor fields in populations at risk of oral cancer. Recently it was shown that genetic alterations in cells brushed from the oral mucosa enables non-invasive detection of precursor lesions in the oral cavity[36], and the potential of keratin 4, cornulin or any of the other identified proteins for such an application might also be investigated.

The next challenge in this field of research relates to the interventions that might relevant when individuals at risk of local relapse are identified. Surgery is not an option when the field is not visible macroscopically, and radiotherapy is not indicated. The most logical intervention would be a change in the watchful-waiting policy. At present treated HNSCC patients visit the outpatient center generally for five years, starting with a visit every six weeks with gradually increasing intervals, till once per year after two years. Patients with precursor fields at high risk of progression should likely be followed lifelong with frequencies of once per three months. The head and neck surgeons might also inspect the mucosal areas at risk in more detail, and decide to biopsy any visible change or excise these immediately. More convenient or perhaps more effective might be to develop a tertiary prevention strategy. Novel experimental approaches have been described to eradicate precursor lesions or to prevent progression of premalignant changes. Topical application of black raspberry gel has shown to induce histological regression and a decrease in the presence of allelic loss.[37, 38] Further, the use of the adenovirus ONYX-015 by oral rinse also demonstrated histological regression and a decrease in mutated p53 expression as detected by immunostaining.[39] These are promising and feasible novel interventions that await further evaluation. Moreover, the genes that are involved in the earliest phase of carcinogenesis are well known, and drugs targeting these genes/proteins might be effective as well.

Taken together, the work in this thesis describes different approaches to discover and validate protein biomarkers for head and neck cancer. It demonstrates that, besides outstanding technological resources, sophisticated study design with proper sample selection and representative validation patient cohorts are indispensable for biomarker discovery. This work has provided new input for the early detection of head and neck cancer.

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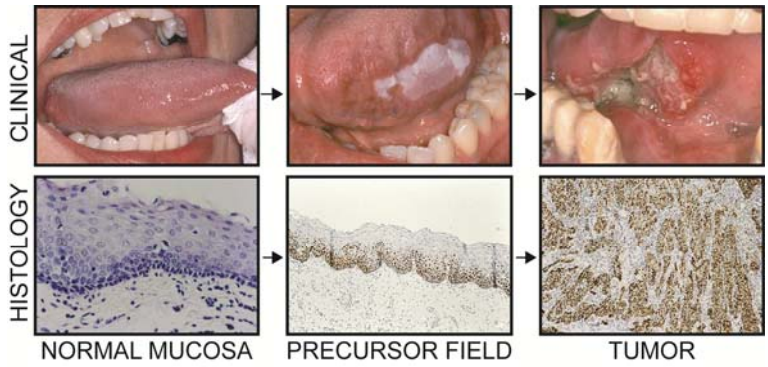
Chapter 8

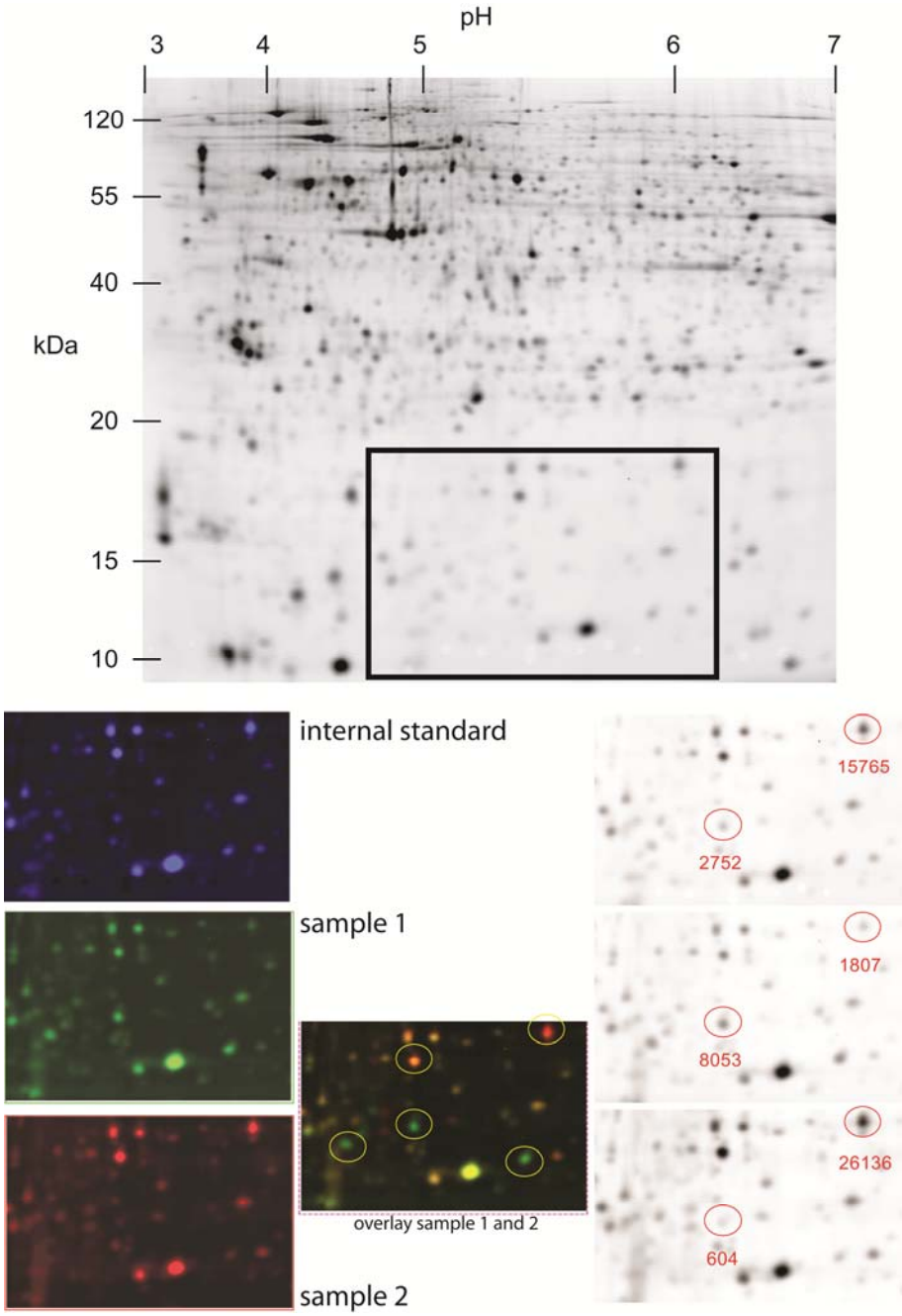
Color figures



CHAPTER 1 - Figure 2. Simplified HNSCC carcinogenesis

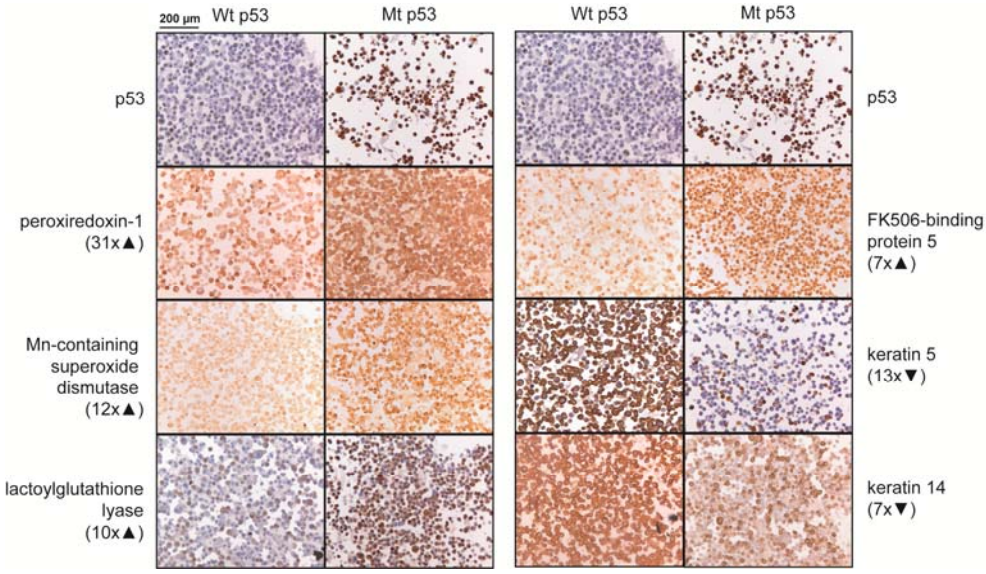
HNSCC's are preceded by precursor fields characterized by:

- allelic loss (100% of all fields)
- p53 pathway disruption (over 50% of all fields, see p53 mutation staining in histological image)
- dysplasia, various grades (the majority of all fields)
- leukoplakia (20% of all fields, see clinical image)
- no other clinical or visible symptoms (80% of all fields)



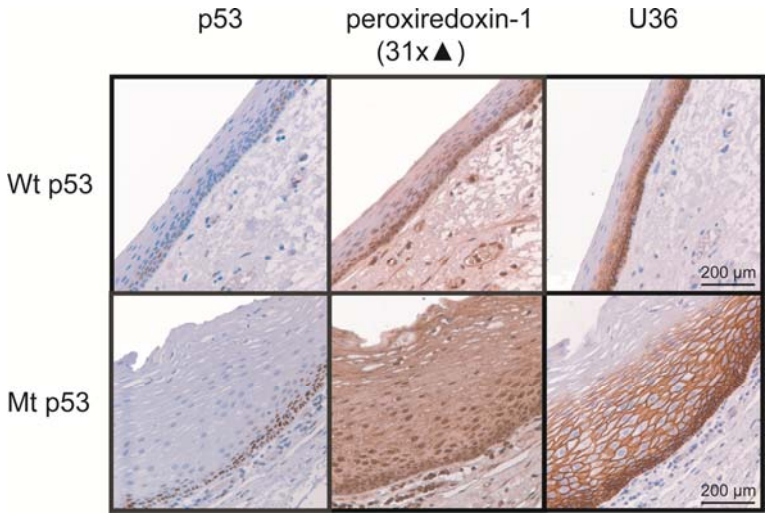
CHAPTER 1 - Figure 3. 2D-DIGE protein separation and quantification

Protein samples are labelled with a fluorescent dye, mixed and separated based on pI and molecular weight. Scanning the gel at the dye-specific wavelengths will produce an image for each corresponding sample. Each spot in the image can be quantified and normalized based on the image of the internal standard.



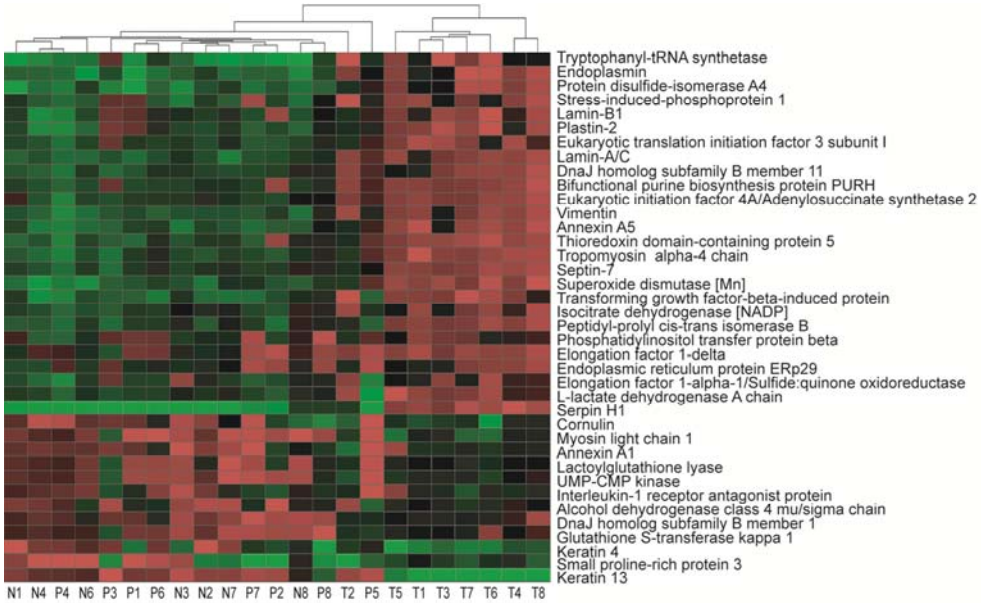
CHAPTER 2 - Figure 4. Verification of differential expression in cell model

Verification by immunohistochemistry of the differential expression of peroxiredoxin-1, manganese-containing superoxide dismutase, lactoylglutathione lyase, FK506-binding protein 5, keratin 5 and keratin 14 for the p53 inactivated cell model. The regulation of each protein, as determined by 2D-DIGE, is stated between brackets. Positive staining is shown in brown; nuclear counterstaining is in blue. The left-hand panels show wildtype p53 cells (Wt p53) and the right-hand panels mutated p53 (Mt p53). Detection of p53 by IHC is only possible in the squamous cell model with dominant negative p53, for which mutated p53 is overexpressed. Upregulated protein expression is shown by more intense staining and/or a higher percentage of positive cells. Downregulation of protein levels is apparent through fainter staining and/or a reduced percentage of positive cells.



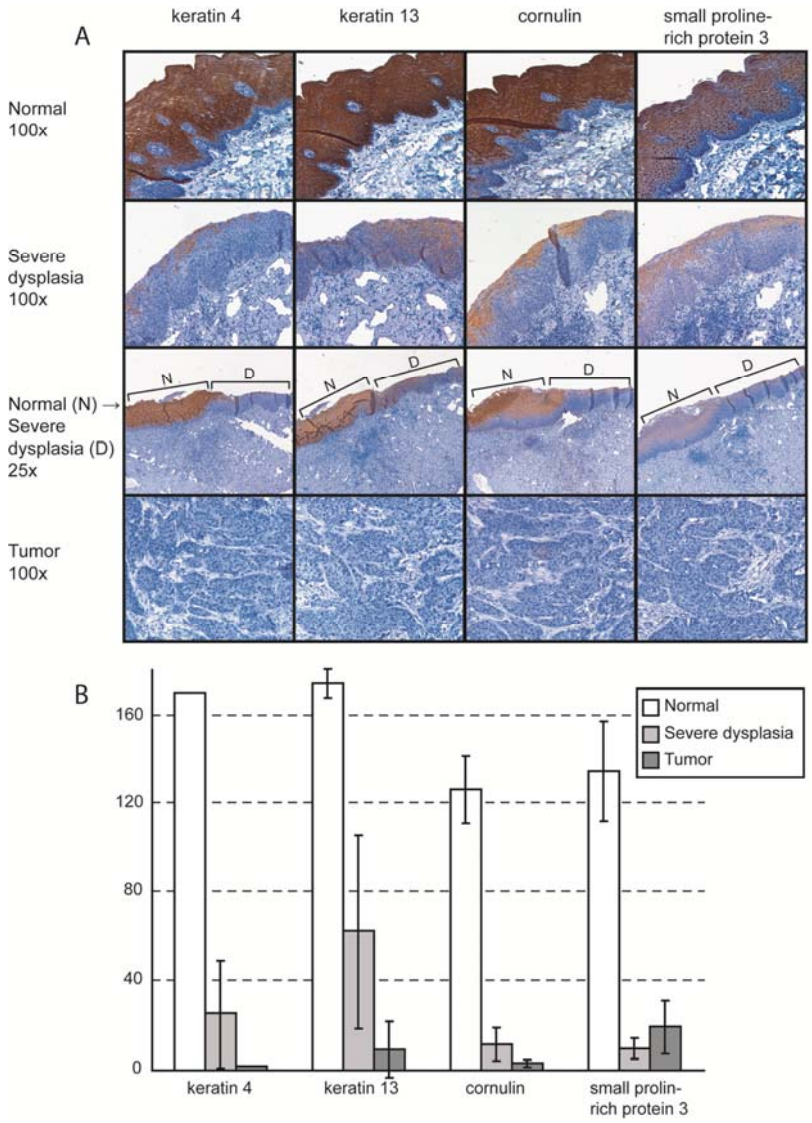
CHAPTER 2 - Figure 5. Immunohistochemical staining of peroxiredoxin-1 on patient tissues

Positive staining is shown in brown; negative counterstaining is in blue. The top panel displays wildtype p53 (Wt p53) and the bottom panel mutant p53 (Mt p53). U36 is a control antibody, directed against CD44v6 that always stains the lower half of the epithelium. Peroxiredoxin-1 clearly demonstrates differential staining of the mucosal cells between normal and mutated p53, in agreement with the over 30-fold increase detected with 2D DIGE. The difference in the appearance between the Wt p53 and Mt p53 tissue sections is mainly due to variation in angle of sectioning.



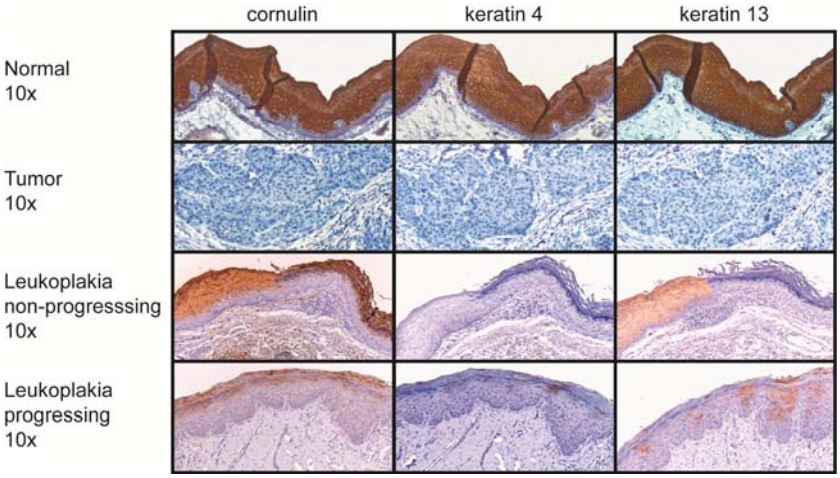
CHAPTER 3 - Figure 3. Heatmap showing hierarchical clustering of patient tissue samples based on the differential protein spots

Green and red colors indicate relatively decreased and increased expression, respectively. Tissue samples are indicated by type (N=normal, P=precancerous, T=tumor) and patient number (1 to 8). All tumors except for one (T2) classify into one cluster, while normal and precancerous fields are intermingled. This classification indicates that the largest differences are found between tumor and mucosal samples independent of whether it was normal or precancerous field tissue.



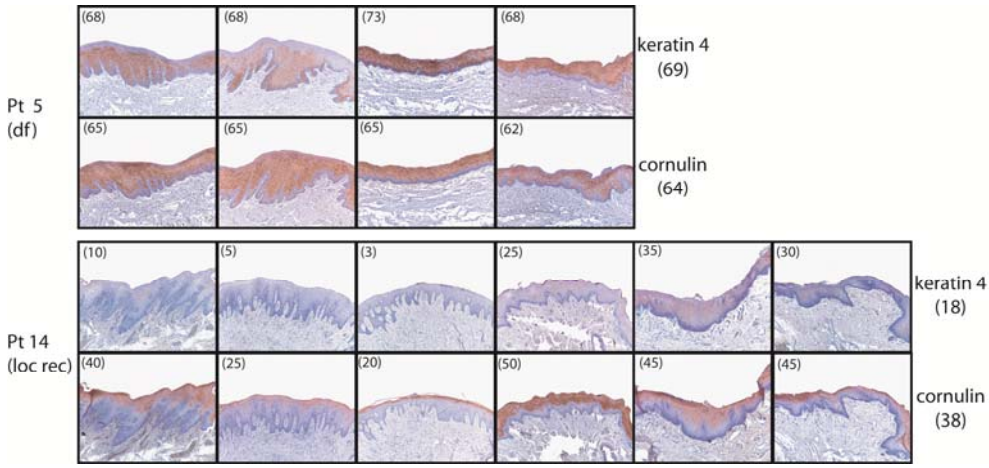
CHAPTER 3 - Figure 4. Validation of differential expression of keratin 4, keratin 13, cornulin and small proline-rich protein 3

A: Typical examples of keratin 4, keratin 13, cornulin and small proline-rich protein 3 staining on normal epithelium, severe dysplasia, and tumor tissue; B: Averaged semi-quantitative scoring results of IHC staining of keratin 4, keratin 13, cornulin and small proline-rich protein 3 on an independent set of 5 normal epithelia, 5 severe dysplasias and 5 tumors. Staining intensity was divided into no staining (=0), moderate staining intensity (=1) and strong staining intensity (=2). Staining results were calculated by multiplying the staining intensity with the estimated percentage of stained cells, resulting in a range from 0 to 200. The expression of all four protein differs significantly between both normal and tumor, and between normal and severe dysplasias ($p<0.01$).



CHAPTER 4 - Figure 1. Examples of cornulin, keratin 4 and keratin 13 staining patterns in normal tissue, tumor tissue and leukoplakia lesions

The most upper panels show the staining pattern of cornulin, keratin 4 and keratin 13 in normal mucosal epithelium and the second upper panels show the staining pattern in tumor tissue. The lower panels show the staining patterns in a non-progressing and progressing leukoplakia lesion. Note the irregular structure of the leukoplakia lesions with sharp demarcations of hyperkeratosis. These images further illustrate the heterogeneous and very diverse staining pattern of cornulin, keratin 4 and keratin 13 in leukoplakia lesions as compared to normal tissue.



CHAPTER 5 - Figure 2. Examples of immunostaining of surgical margins

Representative images of immunostained formalin-fixed paraffin-embedded surgical margins of two patients; one who remained disease-free (df; pt 5) and one who developed a local relapse (loc rec; pt 14). Consensus-score for every margin of both keratin 4 and cornulin is indicated between brackets. Staining patterns can vary slightly throughout one margin. Averaged staining percentages per patient are given between brackets at the right-hand side of the images.

Chapter 9

Samenvatting in het Nederlands

SAMENVATTING

Plaveiselcelcarcinoma in het hoofd-halsgebied (HHPCC) ontstaat in het bekledend slijmvlies van de mond-keelholte en het strottenhoofd. In Nederland worden jaarlijks 2500 nieuwe HHPCC-patiënten gediagnosticeerd en HHPCC is daarmee de zesde meest voorkomende vorm van kanker. Belangrijke risicofactoren voor het ontstaan van HHPCC zijn roken, alcoholconsumptie en infectie met het humaan papillomavirus (HPV). Ondanks de vooruitgang in behandelingsmogelijkheden, is de relatieve vijf-jaars overleving van HHPCC-patiënten nauwelijks gestegen gedurende de laatste 20 jaar. Dit wordt vooral veroorzaakt doordat een groot percentage patiënten zich bij de arts meldt met tumoren in een vergevorderd stadium en doordat bij een groot deel van de patiënten de ziekte na behandeling terugkomt. Vroege opsporing van zowel primaire als recidiverende tumoren kan mogelijk leiden tot een verbetering van de prognose.

HHPCC ontstaat als gevolg van de accumulatie van veranderingen in het DNA van een cel die veroorzaakt worden door externe factoren, zoals bijv. sigarettenrook en alcohol. De carcinogenese (de ontwikkeling van een tumor) van HHPCC verloopt gefaseerd en kan in totaal 15 tot 20 jaar duren. Eerder onderzoek heeft aangetoond dat HHPCC veelal ontstaat in een veld van afwijkende, maar nog niet kwaadaardige, cellen die de eerste genetische veranderingen vertonen. Zo'n veld wordt een precursorveld genoemd en kan vele centimeters groot zijn. Echter, het is meestal niet zichtbaar en geeft ook geen klinische symptomen zoals pijn. Als er meer afwijkingen ontstaan in het DNA van cellen in zo'n precursorveld, kunnen deze cellen veranderen in kankercellen en ontstaat een tumor. Door vroege opsporing van precursorvelden, met name de precursorvelden met een grote kans op maligne ontaarding (progressie), kunnen patiënten met een groot risico op HHPCC geïdentificeerd worden. Deze patiënten komen in aanmerking voor regelmatige controle, zodat tumoren in een vroeg stadium gediagnosticeerd kunnen worden, en ook voor (nieuwe) behandelingen die het ontstaan van kanker kunnen voorkomen.

Door microscopisch onderzoek kan een patholoog bepalen of cellen er afwijkend uitzien (dysplasie) en dit wordt momenteel gebruikt om precursorvelden te identificeren. Deze beoordeling is echter niet geheel betrouwbaar. Het opsporen van genetische afwijkingen in de cellen m.b.v. DNA-technieken is een betrouwbare methode voor het detecteren van precursorvelden, maar DNA-technieken zijn erg bewerkelijk en kunnen daarom niet gemakkelijk geïmplementeerd worden in een diagnostisch laboratorium.

Eiwitten zijn belangrijke moleculen in de cel en verantwoordelijk voor een verscheidenheid aan celbiologische processen. Omdat het DNA als het ware de blauwdruk is voor alle eiwitten in een cel, zullen afwijkingen in het DNA in principe leiden tot afwijkingen in de

eiwitten. Aangezien er relatief eenvoudige en snelle technieken beschikbaar zijn voor het vaststellen van eiwitveranderingen, zouden deze technieken gebruikt kunnen worden voor het identificeren van precursorvelden, mits bekend is welke eiwitten precies veranderd zijn.
Proteomics is een onderzoeksveld dat zich richt op het bestuderen van het proteoom, d.w.z. alle eiwitten die aanwezig zijn in een cel. In het kankeronderzoek worden proteomicsmethoden gebruikt om eiwitbiomarkers te vinden. Eiwitbiomarkers zijn eiwitten waarvan het expressieniveau (de aanwezige hoeveelheid van het eiwit in de cel) veranderd is in bijvoorbeeld cellen in een precursorveld. Daardoor kunnen deze eiwitten gebruikt worden voor diagnostiek. De ontwikkeling van eiwitbiomarkers beslaat verschillende fases en begint met het identificeren van alle veranderingen in eiwitexpressie in bijvoorbeeld een tumor ten opzichte van normaal weefsel waaruit die tumor is ontstaan. Daarna wordt een selectie gemaakt van de meest veelbelovende kandidaat eiwitbiomarkers en deze markers worden gevalideerd in verschillende vervolgstudies. Hierbij wordt gekeken welke eiwitten het meest geschikt zijn voor, in dit geval, het identificeren van precursorvelden. In dit proefschrift zijn verschillende studies beschreven waarin kandidaat eiwitbiomarkers voor de detectie van precursorvelden zijn geïdentificeerd en gevalideerd.
P53 is de naam van een eiwit dat vaak gemuteerd (afwijkend) is in HHPCC en ook in cellen in precursorvelden. In de normale situatie zorgt p53 ervoor dat een cel stopt met delen als er door externe factoren DNA-schade ontstaat. Hierdoor is er tijd om de DNA-schade te herstellen of, als herstellen niet mogelijk is, de cel gecontroleerd te laten sterven (apoptose). Mutaties in het p53-eiwit zorgen ervoor dat p53 inactief wordt en de cel ongecontroleerd blijft delen, ongeacht de aanwezigheid van DNA schade. Dit kan een eerste stap zijn in de ontwikkeling van een kankercel. Voor de studie beschreven in **hoofdstuk 2** hebben we verschillende p53-mutaties nagebootst in een celmodel gebaseerd op cellen uit de mondholte. Het proteoom van de cellen met p53-mutaties hebben we vergeleken met het proteoom van normale cellen uit de mondholte om zo de veranderde eiwitten als gevolg van p53-inactivatie op te sporen. Met behulp van een andere eiwit-detectie-methode, waarbij eiwitten in het weefsel aangekleurd worden, konden we van een aantal van deze eiwitten laten zien dat deze mogelijk goede eiwitbiomarkers zijn voor identificatie van precursorvelden.
Hoewel studies met een celmodel technisch relatief gemakkelijk uit te voeren zijn, vonden we dat het erg tegenviel om de resultaten van een dergelijke studie aan een celmodel te extrapoleren naar de werkelijke situatie in de weefsels van een patiënt. Daarom hebben we voor de studie beschreven in **hoofdstuk 3** geen celmodel, maar weefselbiopten van patiënten gebruikt. Voor deze studie hebben we van acht patiënten normaal weefsel, precursorveldweefsel en tumorweefsel geselecteerd m.b.v. genetische analyses. Het

proteoom van de verschillende typen weefsel hebben we vergeleken om de veranderde eiwitten te identificeren. Voor drie eiwitten, keratine 4, keratine 13 en cornuline, konden we laten zien dat de eiwitexpressie extreem verminderd was in tumorweefsel en ook in een kleine, onafhankelijke serie precursorveldweefsels met een grote kans op progressie. Vanwege deze veelbelovende resultaten, besloten we deze kandidaat eiwitbiomarkers verder te testen.

Leukoplakieën zijn witte laesies in de mond en het is bekend dat in een klein percentage van deze laesies een tumor kan ontstaan. Leukoplakieën zijn dus zichtbare precursorvelden, hoewel vooraf niet te bepalen is welke laesies wel en welke laesies geen progressie naar kanker zullen vertonen. Ook hiervoor wordt momenteel gebruik gemaakt van de dysplasie beoordeling van de patholoog, maar er is veel discussie over de betrouwbaarheid daarvan. Voor de studie in **hoofdstuk 4** hebben we de eiwitexpressie van keratine 4, keratine 13 en cornuline geanalyseerd in 48 leukoplakie-biopten, waarvan er 36 geen progressie hadden vertoond en 12 wel. Deze studie toonde aan dat expressie van keratine 4, keratine 13 en cornuline in leukoplakieën wel zeer afwijkend was, maar niet gecorreleerd aan het ontstaan van tumoren. De meest waarschijnlijke oorzaak hiervoor is dat, specifiek in deze laesies, andere processen de eiwitexpressie van deze biomarkers dusdanig verstoren dat het niet meer mogelijk is om onderscheid te maken tussen de laesies die wel progressie vertonen en de laesies die dat niet doen. In deze serie kon de patholoog met behulp van dysplasie-beoordeling wel goed voorspellen welke laesies maligne ontaarding vertoonden.

Als HHPCC-patiënten geopereerd worden om de tumor te laten verwijderen, kan er een deel van het precursorveld waaruit de tumor ontstaan is achterblijven in de patiënt, omdat dit veld niet te zien is. Uit dit achtergebleven precursorveld kan vervolgens een tweede tumor ontstaan en dit verklaart voor een deel het grote percentage patiënten waarbij de kanker terugkomt. De chirurg zal altijd ruime randen rondom de tumor mee verwijderen (de resectieranden) om hiermee de kans op recidief te verkleinen. Wanneer de resectieranden een deel van het precursorveld bevatten is het te verwachten dat ook een deel van het precursorveld is achtergebleven in de patiënt. Uit dit achtergebleven precursorveld kan weer een nieuwe tumor ontstaan. Voor de studie beschreven in **hoofdstuk 5** hebben we de expressie van keratine 4 en cornuline geanalyseerd in de resectieranden van 46 patiënten, waarvan er bij 23 wel en bij 23 geen tweede tumor was ontstaan. Uit deze analyses bleek dat de expressie van keratine 4 en cornuline in de resectieranden gebruikt kan worden om te voorspellen welke behandelde patiënten een grote kans hebben op het ontwikkelen van een tweede tumor. Deze patiënten kunnen mogelijk extra gecontroleerd worden of in aanmerking komen voor nieuwe behandelingen die erop gericht zijn het precursorveld te

verwijderen en het ontstaan van een tweede tumor te voorkomen. In dit geval bleek de dysplasie-beoordeling van de patholoog geen sterke voorspeller te zijn.
In de laatste jaren is het aantal HHPCC-proteomicsstudies enorm toegenomen. Naast het aantal studies gericht op het identificeren van veranderde eiwitten in HHPCC, is ook het aantal studies waarin kandidaat eiwitbiomarkers gevalideerd worden fors uitgebreid. **Hoofdstuk 6** geeft een overzicht van proteomicsstudies die gedurende de afgelopen jaren zijn gedaan op het gebied van HHPCC en geeft aanbevelingen voor de toekomst van dit onderzoeksveld.

TOEKOMSTPERSPECTIEF

Voor wat betreft HHPCC-proteomicsonderzoek kan nog veel vooruitgang geboekt worden in de verbetering van proteomicstechnologie. Onderzoek naar het gehele proteoom is op dit moment feitelijk onmogelijk, omdat een cel te veel eiwitten bevat om met de huidig beschikbare technieken te analyseren. Bovendien is er vaak per monster te weinig weefsel beschikbaar voor een dergelijk diepgaand onderzoek. Op dit moment bestuderen we dus maar een gedeelte van alle eiwitten. Echter, nieuwe methoden worden ontwikkeld om meer eiwitten te identificeren en om een verscheidenheid aan veranderingen in die eiwitten te meten en te vergelijken tussen verschillende monsters. Daarnaast gaat binnen het biomarkeronderzoek, naast het identificeren van veranderde eiwitten, ook de klinische validatie van kandidaat biomarkers een steeds grotere rol spelen.
Om keratine 4 en cornuline in de kliniek te kunnen gebruiken als biomarkers voor HHPCC precursorvelden in behandelde patiënten, zijn uitgebreidere validatiestudies nodig, waarbij meerdere klinische centra betrokken zijn. Vervolgens is het belangrijk om het klinische beleid vast te stellen voor patiënten met een groter risico op tweede tumoren. In eerste instantie zullen deze patiënten mogelijk frequenter voor controle naar het ziekenhuis moeten komen. Tevens zullen er in de toekomst nieuwe behandelingen ontwikkeld moeten worden waarmee op een milde wijze precursorvelden verwijderd kunnen worden. Verschillende mogelijkheden hiervoor zijn al beschreven en moeten verder getest worden.
Verder kan onderzocht worden waarom het expressieniveau van deze eiwitten verandert en of ze eventueel ook voor andere applicaties of in andere populaties dan behandelde HHPCC-patiënten als biomarkers gebruikt kunnen worden. Naast de eiwitten die gevalideerd zijn in de studies beschreven in dit proefschrift, zijn er nog veel andere eiwitten geïdentificeerd als potentiële biomarkers (hoofdstuk 3), waarvoor ook vervolgstudies gewenst zijn.

Samenvattend, dit proefschrift beschrijft onderzoek waarbij op verschillende manieren eiwitbiomarkers voor HHPCC zijn geïdentificeerd en gevalideerd. Daarbij zijn keratine 4 en cornuline gevonden en in een eerste klinische validatiestudie aangemerkt als veelbelovende biomarkers voor precursorvelden. Daarnaast zijn er nog tientallen andere eiwitten aangemerkt als kandidaat eiwitbiomarkers. Het werk beschreven in dit proefschrift heeft bijgedragen aan de vroege opsporing van primaire en met name secundaire HHPCC en zal in de toekomst hopelijk leiden tot een verbetering in de prognose van HHPCC-patiënten.

Curriculum Vitae and publications

CURRICULUM VITAE

The author of this thesis was born on the 25th of November 1980 in Culemborg. She finished high school at the Prins Willem Alexander College in Leerdam in 1999. In the same year she started her bachelor studies in Biomedical Sciences at the VU University in Amsterdam and in 2002 continued with a master program in Oncology. During her studies she worked as a trainee at the Pathology Department and the Human Genetics Department of the VU University Medical Center (VUmc). Also, she worked on a research project at the Pathology Department of the Queens University Hospital in Belfast.

After her graduation she started her PhD training at the Biomolecular Mass Spectrometry and Proteomics Group of the Utrecht University in collaboration with the Tumor Biology section of the Otolaryngology/Head-Neck Surgery Department of the VUmc. Supervised by Prof. dr. A.J.R. Heck, Prof. dr. R.H. Brakenhoff and Dr. M. Slijper she worked on the discovery and clinical validation of protein biomarkers for head and neck cancer. The results of this PhD research project are described in this thesis. In 2009 she started as a Post-doc at the OncoProteomics Laboratory of the Medical Oncology Department of the VUmc.

PUBLICATIONS

Buffart TE, Carvalho B, Hopmans E, Brehm V, Kranenbarg EK, **Schaaij-Visser TB**, Eijk PP, van Grieken NC, Ylstra B, van de Velde CJ, Meijer GA. *Gastric cancers in young and elderly patients show different genomic profiles.* J Pathol, 2007;211:45-51

Schaaij-Visser TB, Brakenhoff RH, Jansen JW, O'Flaherty MC, Smeets SJ, Heck AJ, Slijper M. *Comparative proteome analysis to explore p53 pathway disruption in head and neck carcinogenesis.* J Proteomics, 2009;72:803-14

Schaaij-Visser TB, Graveland AP, Gauci S, Braakhuis BJ, Buijze M, Heck AJ, Kuik DJ, Bloemena E, Leemans CR, Slijper M, Brakenhoff RH. *Differential proteomics identifies protein biomarkers that predict local relapse of head and neck squamous cell carcinomas.* Clinical Cancer Research, in press

Saydam O, Senol O, **Schaaij-Visser TB**, Pham TV, Piersma SR, Stemmer-Rachamimov AO, Wurdinger T, Peerdeman SM, Jimenez CR. *Comparative protein profiling reveals minichromosome maintenance (MCM) proteins as novel potential tumor markers for meningiomas.* J Proteome Res, in press

Schaaij-Visser TB, Bremmer JF, Braakhuis BJ, Heck AJ, Slijper M, van der Waal I, Brakenhoff RH. *Evaluation of cornulin, keratin 4, keratin 13 expression and grade of dysplasia for predicting malignant progression of oral leukoplakia.* Oral Oncology, in press

Schaaij-Visser TB, Brakenhoff RH, Leemans CR, Heck AJ, Slijper M. *Protein biomarker discovery for head and neck cancer.* Submitted on invitation to Journal of Proteomics

Dankwoord

"Oh the people I've met are the wonders of my world…"♫
Adele, uit Hometown Glory (19, 2008)

Nu vijf jaar, bijna 12.000 fietskilometers en vele "wonderlijke" ontmoetingen later en het boekje is af! Juist de ontmoetingen met bijzondere mensen hebben direct en ook indirect bijgedragen aan de totstandkoming van dit proefschrift en het veraangenamen van mijn promotietijd. Iedereen die hierbij betrokken is geweest, lang of kort, dichtbij of vanaf een afstand, inhoudelijk of randvoorwaardelijk, wil ik hartelijk bedanken!

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