MORPHOMETRY AND SQUAMOUS CELL HYPERPLASIA OF THE LARYNX

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MORPHOMETRY AND SQUAMOUS CELL HYPERPLASIA OF THE LARYNX

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INTRODUCTION

For the definition of the so-called precancerous squamous cell lesions of the laryngeal mucosa, there is little agreement among the authors. Part of this agreement results from the use of inconsistent terminology in describing the histological changes seen in the laryngeal mucosa. Kleinsasser[4] was one of the first to insist on a classification of precancerous changes in the larynx based on the degree of atypical changes of the epithelium. From retrospective clinico-pathological analyses it appears that simple hyperplasia of the squamous epithelium may be the initial morphological alteration from which a carcinoma in situ and eventually an infiltrating carcinoma may develop[4].

Whether the epithelial hyperplasia is simple, with or without atypia, or carcinoma in situ can only be determined by histological examination of adequate and representative biopsy material, obtained by means of (micro-)laryngoscopy. In practice the degree of atypia - the most important histological criterion - has to be assessed accurately, because as was shown in several retrospective clinical analyses[2 5 6], the higher the degree of atypia, the worser the prognosis. Adoption of an uniform classification system with well-defined criteria, including the degree of atypia should be aimed at. Although this was generally felt at the Centennial Conference on Laryngeal Cancer (Toronto, 1974), no unanimous nomenclature was accepted.

An exact histopathological diagnosis, however, is required for the treatment of epithelial hyperplasia. In our institutions we have used since 1970, Kleinsasser's three grade classification: class I, simple squamous cell hyperplasia; class II, squamous cell hyperplasia with atypia; class III, carcinoma in situ. Already in 1963 Kleinsasser

showed[5] that this classification carries prognostic significance, which has been confirmed by two other studies, both including a large series of patients[2 6]. The results of these studies are also reflected in our management policy. When a class I or class II lesion is reported by the pathologist no further treatment is given but the patient is merely followed-up. Class III lesions by large majority received a full course of radiotherapy; in recent years an endoscopic CO_2 laser surgery or only endoscopic microsurgical removal have also been used.

In routine histopathological practice, however, problems are encountered in the use of Kleinsasser's classification, particularly in the distinction between class II and class III. This is not surprising as it is well kwown that consistent histopathological classification of epithelial lesions of the larynx is difficult in general, as these lesions may show a continuous spectrum from normal to neoplastic epithelium. Serious inconsistencies in diagnoses have been described, between observations of pathologists on the same microscopical slide and also between the observations of the same pathologist on one slide at different instances[1 3 7].

In order to achieve a more objective and reproducible classification of the laryngeal lesions, especially hyperplasia with atypia (class II) and carcinoma in situ (class III), morphometry was used. The discriminating morphometrical parameters were selected from a learning set consisting of class II and class III lesions from patients followed-up for a mean period of 8.4 years, (a) to differentiate in an objective reproducible manner class II and class III lesions and (b) to identify those patients in the class II lesions, who had a poor prognosis.

The numerical decision rule derived from these studies was then evaluated on a test set of a new series of biopsy specimens, to identify the prognostic significance of this rule for individual patients.

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DIAGNOSTIC PROBLEMS IN SQUAMOUS CELL HYPERPLASIA OF THE LARYNX.

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CLASSIFICATION

Introduction

The clinical management in case of carcinoma in situ of the laryngeal epithelium generally is a full course of radiotherapy. Alternative treatment methods as cordectomy, laser surgery or only endoscopic microsurgical removal are also applied; the policy depends on the site and extent of the lesion and the age of the patient. Patients with

squamous cell hyperplasia of the vocal cords are merely followed. The treatment policy as to patients with laryngeal squamous cell hyperplasia and atypia theoretically is the same, but many patients, at revision of the biopsy assigned to this class, were found to be irradiated on the assumption at the time that it concerned a lesion with carcinoma in situ (50). An exact histopathological diagnosis, based on a clear classification system, is really necessary for an adequate treatment of epithelial hyperplasia.

In the literature many different classifications of epithelial hyperplasia were found. A perusal of approximately two dozen papers published between 1940-1960 reveals an extremely variable use of histological criteria. In 1950 serious efforts began in an attempt to distinguish between different precancerous epithelial lesions and to assess the frequency with which invasive carcinoma actually followed these abnormalities.

Comparison of the investigations on hyperplastic squamous cell lesions of the larynx is complicated by the inconsistency of the nomenclature and definitions used by the various workers, ranging from near normal epithelium with mild (hyper-) keratosis to severe atypia or dysplasia (mild, moderate, severe) and carcinoma in situ [5 13 15 19 23 26 27 37 39 41 43 50 55 61 63 69].

The question in the general problem of cancer is under what condition and when will the pathological alterations of the tissue develop into true malignancy. The evaluation of the outcome for various groups of patients with abnormal laryngeal mucosal changes is difficult because of - as already mentioned - different classifications used and differences in the length of the follow-up and the clinical management of these lesions. An impression of the probability that invasive carcinoma will develop from the various types of squamous cell lesions may be obtained from the figures reported by several authors (Table 1). When only the lesions with atypia are taken into account 2.7% to 28.6% is found to progress to invasive carcinoma.

At the Centennial Conference on Laryngeal Cancer (Toronto, 1974) the workshop no. 2 panel was devoted to premalignant laryngeal lesions, carcinoma in situ and superficial carcinoma[58]. At this conference the following classification was proposed, but not unanimous accepted: 1. keratosis, 2. keratosis with atypia, 3. carcinoma in situ, 4. carcinoma with micro-invasion.

Table 1. Reported series of patients with keratosis, hyperplasia, with or without atypia, who finally developed a laryngeal carcinoma.

Authors	Number of cases	Number of cases of laryngeal carcinoma without atypia	%	with atypia	%	Total number of cases carcinoma	%	Remarks
Putney, O'Keefe 1953, (61)	62	-	-	-	-	21/62	33.8%	Lack of specific pathologic criteria
McGavran et al. 1960, (55)	84	1/66	1.5%	2/18	11.1%	3/84	3.6%	3 cases were excluded, because an infiltrating ca developed within 6 months
Gabriel, Glyn-Jones 1962, (26)	30	0/13	0.0%	1/17	5.9%	1/30	3.3%	Length of follow-up was not specified
1973, (27)	105	3/50	6.0%	4/55	2.7%	7/150	6.6%	Not indicated whether the developed ca's were in situ or invasive
Norris, Peale 1963, (54)	116	1/30*	3.3%	5/86	5.8%	11/116**	9.5%	* 1/30: 1 patient developed a "superficially invasive carcinoma" ** 11/116: 5 invasive ca, 4 in situ ca, in 2 the presence of invasion was equivocal
Doyle et al. 1977, (20)	28*	-	-	-	-	3/28	10.7%	* 28: in situ carcinoma
Henry 1979, (37)	43	1/29	3.4%	4/14*	28.6%	5/43	11.6%	* 4/14: 3 invasive ca, 1 in situ ca
Crissman 1979, (15)	92	0/76	0.0%	3/16*	18.7%	3/92	3.3%	* 3/16: grade III or severe keratosis
Hellquist et al. 1982, (36)	161	2/98	2.0%	12/63*	19.0%	14/161	8.7%	* 12/63-3/24-9/39; 3/24: originally moderate dysplasia; 9/39: severe dysplasia and in situ ca

Ad 1. Keratosis was described as an epithelial hyperplasia with an orderly maturation sequence, with normal cellular cytology and architecture and some degree of surface keratinization, be it orthokeratotic or parakeratotic (the presence of indentifiable nuclear remnants in the keratotic layer). It is a histological description and not necessarily a diagnosis. The term hyperkeratosis is erroneous[37], because it describes exuberant keratinization of the surface of the vocal cord, but the true vocal cord is normally non-keratinized.

Ad 2. In keratosis with atypia there is some degree of either cellular atypia or disturbance of maturation sequence. The atypia may vary in degree from slight to great and could be graded mild, moderate or severe.

Ad 3. Carcinoma in situ - this term was introduced by Broders in 1932[12] - can be defined as a neoplastic process in which the normal histological structure of squamous epithelium is replaced by cells, which show morphologically all the characteristics of neoplastic cells, but do not show invasive growth. All acceptable cases of carcinoma in situ exhibit loss of stratiform differentiation, altered polarity of cellular orientation and cellular atypia. The absence of a distinct and uniform basal cell layer and the occurence of anisonucleosis throughout the thickness of the epithelium are the most convincing features to the observer[58].

Disagreement arises when less than the full thickness of the epithelial layer is altered. It is generally recognized that some flattening of cells can occur in the superficial layers[5]. Fechner[23] defined carcinoma in situ however, as a full tickness epithelial replacement by atypical cells, whereas the diagnosis of laryngeal atypia was applied to any lesion with atypical cells short of full thickness involvement. In the latter lesion there is maturation or flat superficial cells which may or may not keratinizing.

Ad 4. Micro-invasive carcinoma, although not exactly defined in literature, refers to carcinomas which invade through the basement membrane; the depths of invasion is 2 - 3 millimeter. Batsakis[5] stated that in situ carcinoma and micro-invasive carcinoma appear to behave in a similar biological manner and if coexisting invasive carcinoma can be eliminated, the two lesions may be treated alike.

Every pathologist of the workshop no. 2 panel at the Centennial Conference on Laryngeal Cancer[58] agreed that the term leukoplakia

should be abandonned as a definitive pathological diagnostic term. This term has caused considerable confusion in the past. For oral precancerous lesions leukoplakia is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease, and this term is unrelated to the absence or presence of dysplasia[82].

Kleinsasser's classification

Although a reliable assessment of the various reported classifications and patient studies is difficult, because of differences in histopathological criteria, in terminology and length of the follow-up period, it is obvious that squamous cell lesions with atypia, generally show a more unfavourable prognosis (Table 1) as to the larynx, than lesions do without atypia.

Kleinsasser[41 43] was one of the first to present a classification based on the degree of atypical changes of the epithelium. The lesions fall into three classes according to the internal structure of the epithelium. The following morphological criteria - with modifications of Delemarre[18 19] - are used: class I, simple squamous cell hyperplasia, thickening of the epithelium with a regular architecture. There is a parakeratosis. Basal cell hyperplasia might occur. Mitoses are rare and only found in the basal cell layer (figure 1b, chapter III). Class II, squamous cell hyperplasia with atypia, shows all the characteristics of class I and in addition atypia. Besides parakeratosis there is dyskeratosis (premature or inappropriate development of cytoplasmic keratin in cells located in the prickle layer and the basal layer). A moderate number of mitoses are seen, some of which may be atypical. Atypia denotes to individual cells with nuclear aberrations. The nuclei may be enlarged, irregularly shaped, or may have abnormal staining. There might be a significant loss of polarity. The atypia does not involve the full height of the epithelium (figure 1c, chapter III). Class III, carcinoma in situ, manifests the generally accepted morphological characteristics of carcinoma with the exception of invasive growth: there is a full thickness epithelial replacement by atypical cells. Mitoses are frequent and not limited to the basal cell layer (figure 1d, chapter III). Independent of the category in which the lesion is classified, subepithelial changes consist in edema and cellular inflammatory reaction.

Both Delemarre[18 19] and Lubsen[50] have demonstrated by means of retrospective clinico-pathological analyses that this classification has

prognostic significance: the risk of future development of carcinoma increases from class I to class III (Table 2). In all three studies those patients were excluded in whom within one year from the first biopsy, an infiltrating carcinoma was diagnosed on the assumption that probably the first biopsy has not been representative of the lesion.

Table 2. The number of non-treated class I, class II and class III patients, who developed an invasive carcinoma.

	Class I	Class II	Class III	mean interval
Kleinsasser (44), 1963	5/61	1/ 5	18/20	6 yr
Delemarre (18), 1970	3/20	6/26	4/ 8	2½ yr
Lubsen (50), 1980	1/23	5/18	1/ 3	5½ yr

The clinical management is based on this classification, which means that when class I or class II is reported by the pathologists after removal of the entire lesion, no further treatment is given and the patient is merely followed-up. To the majority of patients with class III lesions radiotherapy is applied.

Diagnostic procedures

In practice the problems encountered in the use of Kleinsasser's classification are found in the differential diagnosis between class II and class III. An exact classification is required because of a different management policy for class II and class III lesions.

The problem of inconsistency in diagnoses between various pathologists has been mentioned by Conley. In 1964 he sent the same slide to four pathologists experienced in laryngeal histology and the reports yielded various results (from hyperkeratosis to cancer of the larynx). For the classification of simple keratosis and keratosis with atypia, Gabriel[28] did a similar experience when two pathologists re-examined 21 of these kinds of lesions. The pathologists did not agree on many diagnoses concerning simple keratosis and keratosis with atypia.

A distinction between epithelial hyperplasia, with or without atypia and carcinoma in situ, can only be determined by histological examination of an adequate biopsy. In that way the ear-nose-throat surgeon is

in a favourable position because early mucosal changes are clinically recognizable, as they cause symptoms such as voice change and hoarseness. According to the pathologist Crissman[16], the classification of changes in squamous epithelia with worrisome atypia, but without invasive growth, is a problem in all upper aero-digestive tract sites. The laryngeal glottis can serve as a model for the study of squamous mucosal changes; the glottis is relatively easy to biopsy.

An important advance in early diagnosis of lesions of the vocal cords has been introduced by Kleinsasser in 1964[46] by the microlaryngoscopic technique. This method is today greatly facilitated by use of microlaryngoscopy under general anaesthesia with jet ventilation[38]. Obstruction to the operating field is minimal, because the tube lies posteriorly in the posterior commissure, leaving an excellent view of both vocal cords and the pulmonary ventilation during general anaesthesia is adequate[76].

Selection of a representative biopsy site can be made with toluidine blue dye, which is lightly painted over the area and then sponged off with saline. In the area taking up the dye most strongly the biopsy specimen should be obtained[51 72]. Exfoliative cytology may also be a diagnostic adjunct. It should never replace histological examination[52 59] because of a high percentage of false negative results in laryngeal malignancies[25] and it has proved to distinguish inadequately between severe dysplasia and invasive carcinoma[9]. However, both methods have not been generally accepted.

It is essential to have close cooperation between the clinician and the pathologist to minimize the problems of non-representative biopsies. Patologists and laryngologists should realize that the diagnosis of a pathologist can only be based on the picture of the slides from the biopsy that have been send to him[18]. A number of representative biopsies from several areas may be necessary to establish the true nature of the lesion.

An adequate biopsy should include mucosa of the whole lesion and some adjacent submucosal tissue with proper orientation. Multiple sections of the entire mucosal surface should be examined with special attention to the margins[8]. Via immunoperoxidase techniques using involucrin staining abnormal differentiation of the hyperplastic laryngeal epithelium may be identified[40]. In the experience of Bauer[7] and Pesch et al.[60] in situ carcinoma in a laryngeal biopsy specimen is almost

always a sign of invasive carcinoma. This is particularly true in biopsies from other sites than the true vocal cord.

With regards to the aforementioned there is need for a more objective and reproducible classification. To obtain this, several additional morphological methods can be performed on the biopsies, such as electron microscopy, photometry and morphometry.

ADDITIONAL MORPHOLOGICAL METHODS

Electron microscopy

Electron microscopic studies have been carried out on the mucous membrane in laryngeal hyperplasia and laryngeal carcinoma. Schenk[65] examined dyskeratotic cells in squamous cell carcinoma of the larynx. These cells were characterized by premature, abnormal and individual keratinization of the malignant keratinocytes. In another electron microscopic study, Schenk[66] found the basement membrane to be very thin and discontinuous or absent from extensive areas of the tumor-stroma junction in case of invasive laryngeal carcinoma. That disruption should nevertheless not be regarded as an ultrastructural criterion for tumor invasion in laryngeal carcinoma.

Sugar[73 74] investigated the fine structural changes as seen in the earliest stages of invasive growth. He found before any massive destruction of the basement membrane, cytoplasmic processes originating from epithelial cells penetrating into the connective tissue through gaps in the basement membrane. This phenomenon was referred to by Sugar as micro-invasion at the electron microscopic level, which can also be observed in precancerous lesions.

Bruchmüller and Hanson[13] examined the connection between mucosal surface pattern (classified according to Kleinsasser's principles of microlaryngoscopy[42 46 47]; epithelial hyperplasia diffusa or circumscripta, last of which can be specified due to plain, verrucous or papillary surface pattern) and the rate of malignant transformation of the precancerous lesions, also in a scanning electron microscopic investigation[32]. The so-called "verrucous" or "papillary" keratosis circumscripta (the irregularly elevated hyperkeratotic type) represented on E.M. findings irregularity of cell and tissue surface, but also roughness and keratinization of microvilli. These features seem to be characteristic scanning

electron microscopic pictures in case of precancerous lesions, in which signs of atypia were proved histologically.

Meyer et al.[57] found with a quantitative electron microscopic analysis of the keratinizing epithelium of normal hard palate, a decrease in density of mitochondria, membrane-bound organelles and free ribosomes from stratum basale to stratum granulosum. Constituents which are considered key structures for the process of keratinization (bundled cytoplasmic filaments, keratohyalin granules and keratinosomes) increase in volumetric density between either stratum basale and stratum spinosum or between stratum spinosum and stratum granulosum. The cytoplasmic ground substance displays a rather stable density throughout all strata.

Although these findings may provide a more objective classification for class I, II and III lesions, in practice this technique is not useful for diagnosis of these lesions, because of the laboriousness and the chance of sampling error.

Photometric investigations

Gröntoft et al.[31] performed a photometric determination of DNA content and nuclear size using a Leitz scanning photometer, in order to obtain a more objective classification. As was also found by Greisen[30] and Giarelli[29], in dysplastic and carcinomatous laryngeal epithelium, hyperchromatic nuclei are numerous. Hellquist et al.[56] classified histological sections into several groups: hyperplasia with or without keratosis, mild dysplasia; moderate dysplasia; severe dysplasia or carcinoma in situ of the classic type.

In the keratotic epithelia no abnormality of nuclear DNA content or nuclear area and only a slight increase in the same values in hyperplastic epithelia was found[34]. This study did not show photometric differences between moderate dysplasia in patients that later developed severe dysplasia and those that did not.

The photometric pattern of lesions such as severe dysplasia and carcinoma in situ differed distinctly from normal epithelium and resembled that of invasive carcinoma[35]. For all the epithelia with severe dysplasia the nuclear DNA content was elevated and the histograms contained abnormal features; the nuclear area was increased.

Well differentiated forms of severe dysplasia, i.e. with marked keratinization, and lesions in which the nuclear atypia was pronounced -

greatly increased DNA content and nuclear size - were prognostically the most serious with a high recurrence rate and were more likely to develop an invasive carcinoma[35]. The diagnosis of the well differentiated form of severe dysplasia is based mainly on structural alterations, rather than on nuclear atypia.

Hellquist[33 35] concluded that nuclear atypia may be concealed to microscopic examination, especially in the case of well differentiated lesions and that photometry seemed to offer a more reliable assessment of the nuclear atypia.

It seems reasonable to expect that measurement of the DNA content of the cells in these lesions might yield important prognostic information of the excisional biopsies.

Morphometry

Base line data for comparison and better understanding of normal and pathologically altered stratified epithelium, have been furnished by many studies on oral epithelia. They provide detailed quantitative characterization of structural constituents of the epithelium.

Stereological studies on stratified epithelia rely on the assumption that structural homogeneity exists within a particular defined epithelial layer[67]. By obtaining data from within each cell layer, the individual strata can be analysed quantitatively. The process of differentiation can be studied by comparing data obtained from successively higher epithelial layers[81].

Cells at different levels or strata of the epithelium differ from one another structurally. In the basal cell layer of the non-keratinizing squamous epithelium - consisting of one layer of basal cells - the cells are arranged perpendicular to the basement membrane. The differentiation and structural gradients follow a direction from basal to surface layers. Differentiation evolves simultaneously in cells leaving the stratum basale. Therefore, areas of a rather homogeneous state of differentiation can be located reproducible along an axis vertical to the epithelial surface[78]. The cells mature in a regular fashion to become more ovoid in the prickle cell or intermediate cell layer. The nucleus enlarges slightly, assumes a spherical shape and has less dense chromatin. The intercellular bridges (or desmosomes) become quite conspicuous. Finally the cells flatten out in the superficial layer. This layer is comprised of one to three layers flattened cells with small condensed nuclei. Just

below the surface is a granular cell layer; the granules are composed of keratohyaline, the precursor of keratin. During differentiation, cell volume increases between basal and granular layers, whereas the alterations of nuclear volume are less consistent[63 80]. The decrease in n/c ratio (nuclear/cytoplasmic ratio) shows a progressive reduction during differentiation[48 49 57 80]. So the increase in cytoplasmic volume is reponsible for the decrease in n/c ratio during epithelial differentiation.

After characterizing quantitatively the gradient of structural differentiations the next aim of stereological studies of the stratified epithelium of the oral mucosa is to compare the differentiation pattern of normal and pathologically altered epithelium[78]. In pathological epithelia representative areas have to be selected within the strictly defined different layers, because the changes are often focal[48].

Klein-Szanto et al.[48] found remarkable similar differentiation patterns between the basal and surface layers in leukoplakias from the oral mucous membrane of the cheek and the floor of the mouth, for various cytoplasmic and nuclear constituents.

Boyson and Reith[11] and Rigaut et al.[62] were the first to present a morphometrical model for the evaluation of quantitative morphological alteration of the respiratory tract and did so for the nasal epithelium in nickle workers. They staged alterations in metaplasia, dysplasia and carcinoma by evaluation of cells (number, area, maximum diameter, circumference) of the basal layers, using a semi-automatic device. The n/c ratio remained essentially unchanged in different types of metaplastic epithelium, whereas in dysplasia and in carcinoma the n/c ratio showed a slight and pronounced increase respectively. Mean nuclear area was smallest in stratified cuboidal epithelium and largest in squamous epithelium and dysplasia.

No other morphometrical studies using representative photomicrographs and measured on a graphic tablet have been described for multi-layered surface epithelium of the respiratory tract.

The reproducibility of the diagnosis in lesions, which vary from normal to malignancy as a continuous spectrum, can in general be improved with quantitative objective methods[4]. Morphometry, using simple objective parameters, can easily be applied on any routinely processed material. This method has successfully been applied in the tumour pathology of for instance endometrium to support the discrimination between atypical hyperplasia and carcinoma[1 2] and for squamous

cell changes in condyloma acuminatum and dysplasia of the uterine cervix[10] and in normal and carcinomatous squamous epithelia of the cervix[79].

MORPHOMETRY AND THE PRESENT STUDY

In the current study our experience with the classification system of Kleinsasser will be reviewed in a retrospective clinical analysis (chapter II), carried out on patients with squamous cell hyperplasia of the laryngeal epithelium, seen between 1963-1981 in the Free University Hospital and the Antoni van Leeuwenhoekhuis (The Netherlands Cancer Institute), both in Amsterdam. We will attempt to determine whether morphometrical analysis of the epithelial characteristics can contribute to improve the histopathological classification of squamous cell hyperplasia of the larynx. In practice the problems encountered in the use of this classification are found in the area between class II and class III. Therefore in chapter III morphometry will be applied to 50 microscopic slides, routinely processed (15 class I, 15 class II and 15 class III; 5 slides from normal laryngeal epithelium will be added), in order to find features for distinction among the classes and distinction against normal epithelium. This study will focus particularly on the differential diagnosis between class II and class III. The slides for this study are selected on their quality and on their representativity for the class in which the lesion was classified, according to Kleinsasser's classification, without knowledge of the patients' records. The measurements are specifically performed on areas representative for the lesion, as pathological epithelial changes are often focal.

The following features will be used in the assessment of the classification function, discriminating between hyperplasia and atypia (the features will be explained further in chapter III) : 1. mean nuclear area (μm^2), 2. mean nuclear contour index ($NCI = P/\sqrt{A}$), its value increases when nuclear irregularity increases, 3. mean nuclear polarity (radians), a parameter for nuclear axis orientation, 4. total number of nuclei per unit area, a measure for nuclear crowding, 5. maximum width of the epithelium (μm), 6. maximum width of the stratum corneum (μm), 7a. the standard deviation for the nuclear area (a measure for anisokaryose), b. the standard deviation for nuclear contour index (a measure for polymorphia) and c. the standard deviation for nuclear polarity (a measure for irregularity). The features no. 1, 2, 3 and 4 will be measured in the three cell layers (the basal cell layer, the prickle or intermediate cell layer and the superficial cell layer). The quality of the sections, fixed in 10% neutral formalin, does not allow for accurate measurement of the cell size.

In chapter IV the same morphometrical features will be used on laryngeal epithelial hyperplasias and atypias in groups of only untreated patients of class I, II and III with a long term follow-up, from patient material, analysed earlier by Lubsen[50].

To evaluate the results of the learning phase of this study (chapter III and IV), a test study (chapter V) will be applied on another series of biopsy specimens (the test-set).

Finally some closing remarks will be made and lines for further study to achieve a more reproducible classification, will be suggested.

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SQUAMOUS CELL HYPERPLASIA OF THE LARYNX.
(A clinical follow-up study)

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ABSTRACT

According to Kleinsasser's classification 200 patients with squamous cell hyperplasia, seen between 1963-1981, were reviewed histologically. The untreated patients (47%) have been analysed for the incidence of malignant change and the patients with class III lesions (carcinoma in situ) who were treated, were analysed for response to treatment. The mean follow-up period was 8.4 years. Only 2 of the 38 initially untreated patients of class I (simple squamous cell hyperplasia) developed an invasive carcinoma. In class II (hyperplasia with atypia), of 62 patients who were not treated initially, 17 developed a laryngeal squamous cell carcinoma later. Only 6 patients of class III did not receive any treatment initially, and one of these progressed to invasive carcinoma. Almost all other patients with carcinoma in situ (class III) were irradiated. In these patients no evidence of local recurrence was found.

INTRODUCTION

Comparison with reported series is difficult because the terms used to describe abnormal laryngeal mucosal changes vary considerably, as does the clinical management of these lesions and the length of follow-up. In North America usually the classification proposed at the Centennial Conference on Laryngeal Cancer (Toronto, 1974) is being used: keratosis, keratosis with atypia, carcinoma in situ. Others base their classification also on the degree of dysplasia (Hellquist et al.,

1982; Michaels, 1984), which results in 3 groups: group 1, hyperplasia and/or keratosis, with or without mild dysplasia; group 2, moderate dysplasia; and group 3, severe dysplasia and carcinoma in situ. Although the aforementioned classifications use a different nomenclature, they all distinguish three categories and in fact these categories more or less coincide with the classification of Kleinsasser, which has been used traditionally in the Netherlands.

Histopathologically lesions of the laryngeal epithelium with squamous cell hyperplasia can be classified into three classes according to the degree of cell atypia present (Kleinsasser, 1963a; Delemarre, 1970); class I: simple squamous cell hyperplasia; class II: squamous cell hyperplasia with atypia; and class III: carcinoma in situ. There seems to be a correlation between the different classes and the chance that an invasive carcinoma may develop (Kleinsasser, 1963b; Delemarre, 1970).

Our clinical management was based in this classification. In all cases excisional biopsy of the whole lesion was attempted to facilitate histological examination. Patients with class I and class II lesions have been followed-up without treatment. Almost all patients classified as carcinoma in situ (class III) received a full course of radiotherapy. In recent years alternative treatment methods like CO_2 laser surgery or only endoscopic microsurgical removal were also applied, the policy depending on the site and extent of the lesion and the age of the patient. In the present report our experience with the classification system of Kleinsasser in 200 patients has been reviewed and the untreated patients have been analysed for the incidence of malignant change. Those who were treated have been analysed for their response to treatment.

MATERIAL AND METHODS

Histopathology

Kleinsasser's classification (1959, 1963a), with the modification of Delemarre (1970), recognises three categories; class I is simple squamous cell hyperplasia, the epithelium is thickened and there is keratosis. The epithelium shows a regular pattern without any atypia (Fig. 1b, chapter III). Occasionaly mitoses are found in the basal cell layer. Class II, squamous cell hyperplasia with atypia, shows the features of

class I with atypia. A moderate number of mitoses is present. The atypia does not involve the full thickness of the epithelium (Fig. 1c, chapter III). Class III, carcinoma in situ, manifests intraepithelial neoplasia with full-thickness epithelial replacement by atypical cells. Mitoses are frequentand not limited to the basal cell layer (Fig. 1d, chapter III). There is no evidence of invasion.

Patients

This study is based on a retrospective clinico-patholical analysis of laryngeal biopsy specimens obtained from 215 patients with squamous cell hyperplasia seen between 1963-1981 in the Department of Otolaryngology of the Free University Hospital and the Antoni van Leeuwenhoekhuis (the Netherlands Cancer Institute), both at Amsterdam, the Netherlands. Eleven patients were excluded from this analysis because an infiltrating carcinoma was diagnosed within one year of the first biopsy. The first biopsy may not have been representative of the tumour. Four patients were lost to follow-up (all assigned to class I).

The remaining 200 patients were divided into the three classes as follows: class I: 38; class II: 92; class III: 70 (Table I).

Table I. 200 patients with laryngeal squamous cell hyperplasia, classified on the basis of the initial biopsy in class I, II and III (Kleinsasser's classification).

	Class I	Class II	Class III	Total
Male	33	86	60	179 (89%)
Female	5	6	10	21 (11%)
Total	38 (19%)	92 (46%)	70 (35%)	200 (100%)

The highest incidence of laryngeal hyperplasia was found in the fifth, sixth and seventh decades. The youngest patient was 23 and the oldest 85 years of age. The mean age was: 59.8 ± 12.3 years. Of the 200 patients 179 were males (89%, ranging in age from 23 to 85 years (mean: 60.7 ± 11.4 years), and 21 were females (11%), ranging in age from 26 to 76 years (mean: 52.7 ± 17.8 years) (Fig. 2). For class I, II and III, the mean ages were 54, 60 and 62 years respectively.

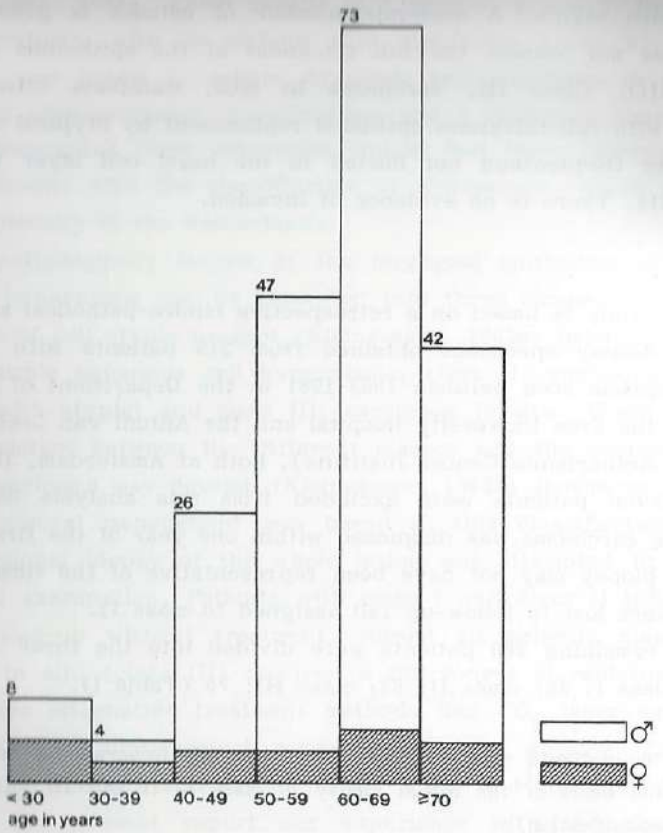

Fig. 2 : Distribution of age and sex of 200 patients with squamous cell hyperplasia of the larynx.

No accurate information on smoking and drinking could be obtained from the available data. However, only 17 patients (8%) were non-smokers; of the smoking-group most patients smoked cigarettes. Most of the patients also consumed alcohol. The presenting symptom was hoarseness.

In the great majority, the lesions were limited to one vocal cord (n=156; 78%). The anterior two-thirds were commonly involved (n=96; 49%). Less frequently the posterior one-third (n=21; 11%) or the whole length of one or both vocal cords (n=78; 40%) were involved. Rarely the lesions extended to or arose from the supraglottic or subglottic regions (n=5; 2½%).

The patients were followed-up for a mean period of 8.4 years

(range: 5-21 years). Patients assigned to class I or class II were seen every 2 months during the first year after the initial biopsy and every 3 and 4 months respectively in the second and third years. From the fourth year the patients were seen every 6 months. Patients with carcinoma in situ (class III) were seen more frequently. Thirty-eight patients with a class I lesion received no treatment initially, but were followed-up. From the 92 patients with a class II lesion 62 have not been treated initially, but 30 patients were irradiated, on the assumption at the time, that they were class III lesions. Out of a total of 70 patients with class III lesions, 62 received initially radiotherapy and 2 were treated by cordectomy. Three were not irradiated because their lesions were at the time diagnosed as class II. Two patients were not treated because, when the first laryngeal biopsy was taken, a primary bronchial carcinoma was diagnosed. Another patient was not treated because of a myocardial infarction.

RESULTS

Patients with class I lesions

Only 2 of the 38 initially untreated patients developed an invasive carcinoma, 2 and 7½ years after the first biopsy. Both patients received a full course of radiotherapy. One of these patients died one year after the completion of radiotherapy with uncontrolled disease in the neck. The second patient was salvaged. Another of the 38 patients was classified as class III, 5 years after the first biopsy and was then treated with a full course of irradiation; this patient is free of disease. Two patients were irradiated on the basis of a second or third biopsy originally classified as class III, but at revision assigned to class II; both patients died (cardiovascular disease; stomach carcinoma).

Patients with class II lesions

Initially untreated patients

Sixty-two out of the total of 92 class II patients (Table I) have not initially been treated. Of this group 17 patients developed an invasive laryngeal carcinoma, from 13 months till almost 10½ years after

the initial diagnosis (Fig. 3).

Three patients (nos. 125, 127, 129) progressed to invasive carcinoma through the carcinoma in situ stage, but were not treated for their class III lesion, because these lesions were at the time classified as class II. All three patients were treated with radiotherapy when the diagnosis of invasive carcinoma was established. Two are doing well. The third patient (no. 129), however, developed metastases to the mediastinum, of which he died despite radiotherapy. Two other patients received radiotherapy when they were proven histologically to have progressed to carcinoma in situ, but developed invasive carcinoma 2½ and 2¾ years after the irradiation (nos. 118, 119) (Fig. 3). In one patient a total laryngectomy and in the other patient a partial laryngectomy was performed and both patients were salvaged. The other 12 patients developed an invasive laryngeal carcinoma without going through an indentifiable carcinoma in situ stage. Ten patients were irradiated and two (nos. 132, 134) underwent a total laryngectomy, one with en bloc radical neck dissection, with post-operative radiotherapy. These last 2 patients both died with recurrent tumour in the neck. Of the 10 irradiated patients 4 patients suffered a local recurrence. Three of these patients (nos. 122, 126, 130) were treated by total laryngectomy and one (no. 133) by partial laryngectomy. One patient (no. 126) died with recurrent tumour in the neck, whereas another (no. 133) died of bronchial carcinoma.

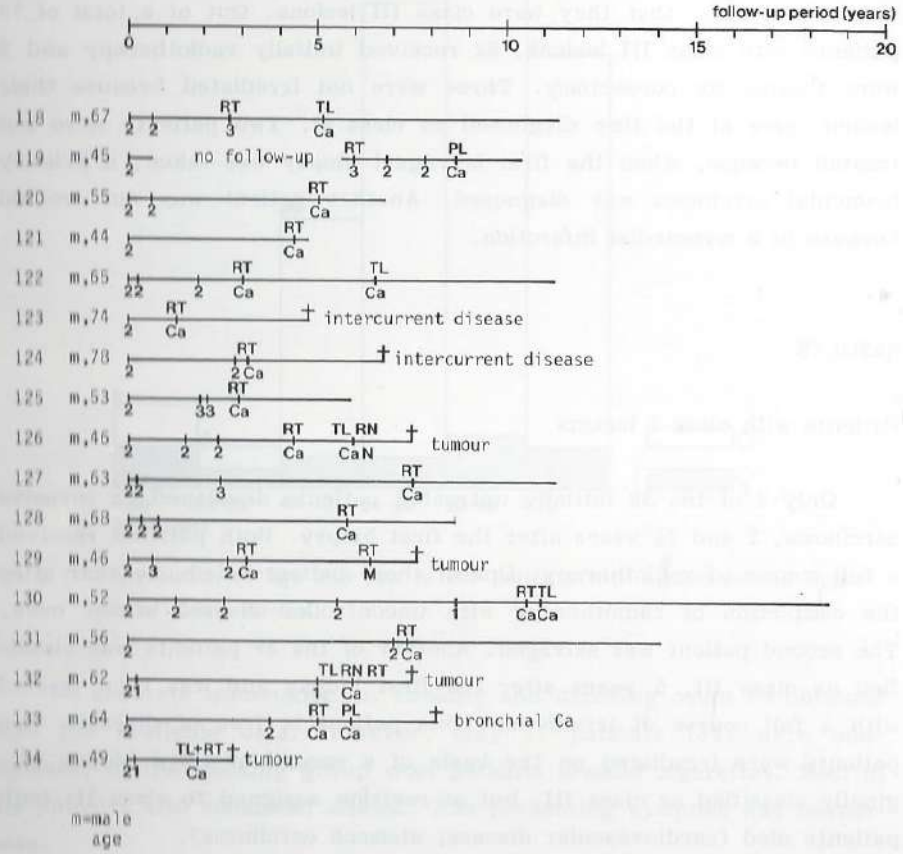

Fig. 3 : Follow-up of 17 initially untreated patients with an original class II lesion (squamous cell hyperplasia with atypia) subsequently developing an invasive carcinoma (=Ca), who received radiotherapy (=RT) or underwent a total laryngectomy (=TL) or a partial laryngectomy (=PL); N = necknode metastasis/M = distant metastasis/ChT = chemotherapy/ RN = radical neckdissection/ 1,2,3 = classification in class I, II or III/ † = died.

Of the remaining 45 initially untreated patients 7 were irradiated on second or third biopsies classified as class II (originally assigned to class III; n=2) or as class III (n=5). All these 45 patients had a favourable clinical course as to the larynx. However, 2 patients died from a bronchial carcinoma.

Initially irradiated patients

Thirty patients classified in class II at revision of the microscopical slides of the initial biopsies have been irradiated initially on the assumption at the time of the original classification, that the biopsy concerned a class III lesion. Two of these patients developed radionecrosis of the larynx, for which a tracheostomy was necessary 14 months after the radiotherapy in one patient (Table II). In the other patient a total laryngectomy had to be carried out more than 11 years after the irradiation. He died from post-operative complications.

Three other patients developed an invasive carcinoma, one patient after more than 5, the second after 8½ years after the first biopsy. These 2 patients were treated with total laryngectomy and were salvaged. The third patient was treated palliatively with chemotherapy, because at the same time as he demonstrated an infiltrating carcinoma - four years from the first biopsy -, metastases to the neck and the mediastinum were diagnosed. He died soon thereafter. Another 2 pa-

Table II. Follow-up of 122 patients who received a full course of radiotherapy as the primary or secondary form of treatment for their lesions initially or during follow-up classified as class II and class III, or who developed an invasive carcinoma later on.

	radiotherapy primarily	radiotherapy secondary			chronic complications	progression to invasive Ca.
		initially classified as:				
		class I	class II	class III		
Class II	30	2	2	-	2: radionecrosis	3
Class III	62	1	7	2	1: persistent edema	2
Invasive Ca.	16	-	-	-	-	4 (recurrence)

tients died from a bronchial carcinoma.

Patients with class III lesions

Initially untreated patient

Six out of the total of 70 class III patients (Table I) initially did not have any treatment for their carcinoma in situ of the larynx (Fig. 4). Two of these patients (nos. 190, 191) were in a poor condition

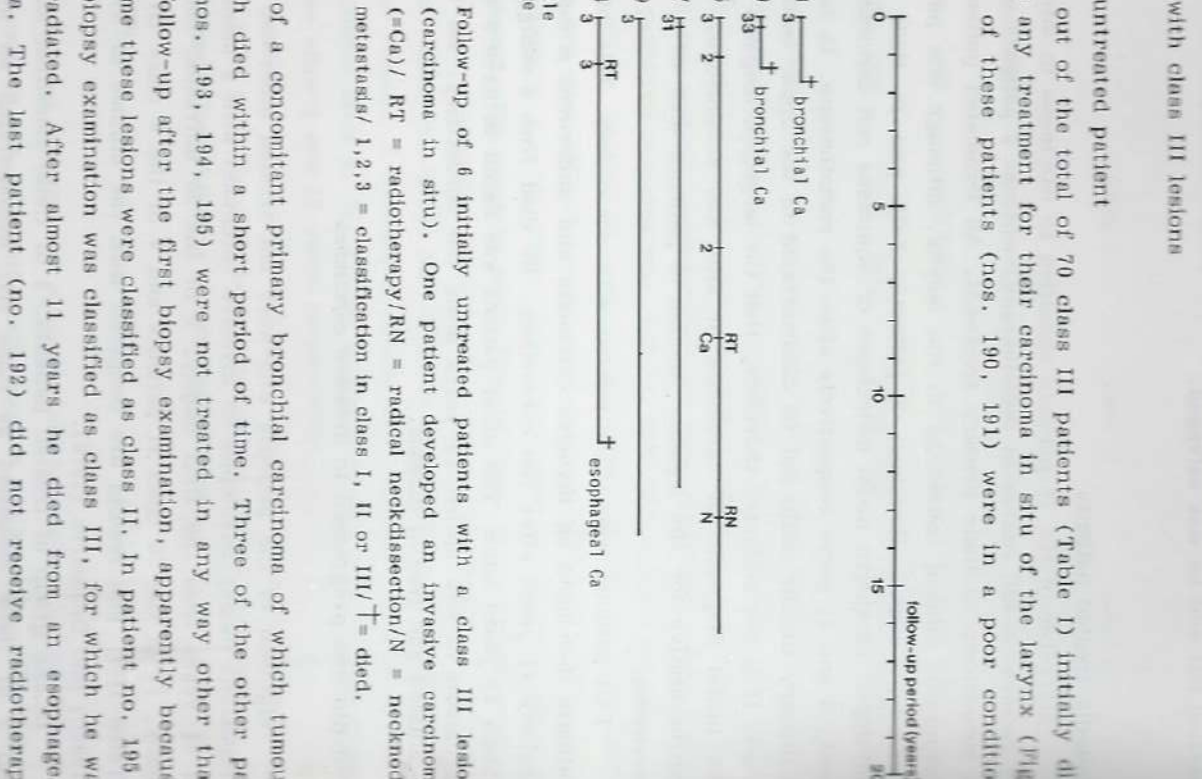

Fig. 4 : Follow-up of 6 initially untreated patients with a class III lesion (carcinoma in situ). One patient developed an invasive carcinoma (=Ca)/ RT = radiotherapy/RN = radical neckdissection/N = necknode metastasis/ 1,2,3 = classification in class I, II or III/ † = died.

because of a concomitant primary bronchial carcinoma of which tumour they both died within a short period of time. Three of the other patients (nos. 193, 194, 195) were not treated in any way other than careful follow-up after the first biopsy examination, apparently because at the time these lesions were classified as class II. In patient no. 195 a second biopsy examination was classified as class III, for which he was then irradiated. After almost 11 years he died from an esophageal carcinoma. The last patient (no. 192) did not receive radiotherapy initially because of a myocardial infarction. On a second biopsy, taken almost one year after the initial biopsy examination, the lesion was classified as class II. However, 8 years after the first biopsy he developed an invasive carcinoma and was irradiated. After 5 years metas-

tases to the neck were found for which a radical neck dissection was carried out and he was salvaged.

Initially treated patients

Sixty-two of the 70 patients classified as carcinoma in situ, initially received a full course of radiotherapy, whereas 2 patients underwent a cordectomy as their primary treatment. All irradiated patients had a favourable clinical course as to the larynx, although one patient had persistent edema. However, 2 patients, both men in their eighties, developed extensive nodal metastases of squamous cell carcinoma in the neck, 3 and 3½ years respectively after the radiotherapy. In both cases a primary tumour could not be demonstrated in the upper aerodigestive tract. It is not unlikely, therefore, that the laryngeal biopsies on which the initial diagnosis of carcinoma in situ was established, were not representative for the lesions. Another 2 patients died of a bronchial carcinoma.

Two patients were first treated with cordectomy. One of these 2 patients developed an invasive carcinoma and underwent a total laryngectomy 4½ years after the cordectomy. He died from a malignant lymphoma 2½ years later. The other patient was found to have a recurrent carcinoma in situ almost 2½ years after the cordectomy. He was then irradiated and free of disease.

Patients who progressed to invasive carcinoma

Eighteen of the patients who were not treated otherwise than by (repeated) biopsy developed an invasive carcinoma with a mean interval of 4¾ years from the first biopsy; 2 of the 35 untreated class I patients, 12 of the 50 class II patients and 4 of the 8 class III patients (Table III). The results are compared with the studies of Kleinsasser (1963b) and Delemarre (1970).

In the group of treated patients, 3 of those classified as class II and 3 of those classified as class III, demonstrated an infiltrating carcinoma later. Together 24 patients developed an invasive laryngeal carcinoma from 15 months till almost 11 years after the first biopsy (mean: 5.4 ± 2.6 years). Sixteen patients received a full course of irradiation as the primary treatment. Four of these patients developed a

Table III. Malignant chance in patients who were not "treated" otherwise than by (repeated) biopsy.

	Class I	Class II	Class III
Kleinsasser 1963	5/61 (8%)	1/5 (20%)	18/20 (90%)
Delemarre 1970	3/20 (15%)	6/26 (23%)	4/8 (50%)
This study 1985	2/35 (6%)	12/50 (24%)	4/8 (50%)

recurrence (Table II); they then underwent a total (n=3) or a partial (n=1) laryngectomy. In 6 patients a primary laryngectomy was undertaken because these patients were irradiated before on a class II or III lesion. In one patient a cordectomy had been performed. One patient received palliative chemotherapy, but he died after a short period.

Out of the total of 24 patients who progressed to invasive carcinoma, 14 are alive without tumour, with a mean follow-up period of 5 ± 3½ years (range: ½ - 13½ years) from the moment an invasive carcinoma was diagnosed. Ten patients died. Four of these died without laryngeal tumour (2: cardiovascular diseases; 1: malignant lymphoma; 1: bronchial carcinoma) after 3½, 3¾, 2½ and 3¼ years respectively. Six patients died from their tumour, 4 because of locally and regionally recurrent laryngeal carcinoma after ¾, 1¼, 1½ and 3¾ years respectively, 2 from distant metastases after ¾ and 5¾ years respectively.

In 2 patients an invasive laryngeal carcinoma never was diagnosed, however, both patients died from nodal metastases in the neck, 3½ and almost 4 years after the first biopsy examination.

DISCUSSION

The highest incidence of squamous cell hyperplasia of the larynx is found in men in the fifth and sixth decades (Delemarre, 1970; Henry, 1979; Hellquist et al., 1982). In the series reported by Delemarre (1970) no significant differences were found between the mean ages of the patients of the three classes, although patients with a hyperplastic lesion of the laryngeal epithelium were younger than patients with an

invasive carcinoma. Hellquist et al. (1982) supposed a gradual development from benign to more severe lesions, because of the higher mean ages of the patients with increasing dysplasia (group I, II, III: 55, 60 and 63 years respectively). In the present study the mean ages of the patients from class I, II and III were 54, 60 and 62 years respectively.

Smoking is found to enhance the risk of laryngeal cancer (Auerbach et al., 1970; Williams and Horm, 1977; Wynder et al., 1956). Besides tobacco, alcohol consumption is a readily identifiable risk factor for laryngeal cancer (Vincent and Marchetta, 1963; Wynder et al., 1976). Hinds et al. (1979) and Wynder et al. (1977) demonstrated that smoking and alcohol consumption appeared to act as independent risk factors. Concerning the amounts of alcohol consumption and smoking no adequate information could be obtained from the available data, but most patients have consumed alcohol and smoked cigarettes.

The lesion was seldom situated outside the vocal cords (Henry, 1979; Hellquist et al., 1982). The anterior part of the cord is most commonly involved. The lesion may be unilateral or both vocal cords are affected (in this study: 75% and 21% respectively).

No definite diagnosis can be based on clinical or microlaryngoscopical criteria alone. A close collaboration between the laryngologist and the pathologist is necessary. Various types of squamous cell hyperplasia can occur adjacent to carcinoma in the same lesion and underscore the necessity for an adequate biopsy (Barney, 1970). In the experience of Bauer (1974) and Pesch et al. (1976) in situ carcinoma in a laryngeal biopsy specimen from sites other than the true vocal cords is almost always a sign of invasive carcinoma. The biopsy should include the most serious lesion present, which determines therapy and prognosis.

In practice the problems encountered in the use of Kleinsasser's classification focus particularly on the differential diagnosis between class II and III. On revision about 1/3 of the cases in the present series classified as class II, were originally assigned to class III, while only a few cases at the time classified as class II, were classified at revision as carcinoma in situ. In consequence about 1/3 of the class II patients received a full course of radiotherapy.

The reproducibility of the classification may improve, when using strict criteria for class II and class III. Moreover, with the use of objective diagnostic methods as for instance photometry (Hellquist et al., 1984) or morphometry (Olde Kalter et al., 1985), the degree of

atypia as reflected in changes of cells and nuclei, can be more accurately assessed.

Patients with class I and class II lesions are only followed-up. When a class III lesion is reported by the pathologist, in most cases a full course of radiotherapy is given. The failure rate for patients classified initially or later on in class III, who received radiotherapy as an initial or secondary form of treatment, was 3%; 2 out of 72 patients (Table II) developed an invasive carcinoma. The recurrence rate reported in other series of patients with carcinoma in situ who were initially irradiated range from 4.5% (Doyle et al., 1977) to 51% (Miller, 1970, 1974). For irradiated patients of class II the results were less; 3 of the 34 patients progressed to invasive carcinoma (9%).

Depending on the age of the patient and the site and extent of the lesion, our alternative treatment methods are CO_2 laser surgery or endoscopic microsurgical removal alone. Hintz et al. (1981) reported a watchful policy on 27 patients with in situ carcinoma. Ten patients' in situ carcinomas have not become invasive. It concerned, however, patients with short-term follow-up (mean: 50 months). Total laryngectomy was required twice as often in the group of patients who were treated expectantly as in the group treated immediately.

It is important to mention that even with an accurate follow-up schedule, 6 patients died from their laryngeal tumour, in some cases with an unexpectedly aggressive course. Nine patients died of a bronchial carcinoma. The risk of developing a second primary tumour in patients with carcinoma in situ is 15%, about equal to the risk in patients with glottic cancer (de Vries and Snow, 1985).

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MORPHOMETRY OF SQUAMOUS CELL HYPERPLASIA OF THE LARYNX.

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SUMMARY

The histopathological diagnosis of squamous cell hyperplasia of the larynx is very subjective. Since morphometry is highly reproducible, this method was applied to routine processed slides of 45 such lesions to assess objectively the epithelial characteristics. In each case measurements of nuclei of 50 cells in the basal, intermediate, and superficial cell layers were carried out. The data were analysed statistically. The findings suggest that morphometry may be helpful for the histopathological classification of squamous cell hyperplasia of the laryngeal mucosa.

INTRODUCTION

Considerable uncertainty and controversy still surround the so called precancerous lesions of the laryngeal mucosa. These lesions develop from squamous epithelium and the changes in this epithelium include hyperplasia, keratosis, and atypia. Proper diagnosis is considerably hindered by the lack of an internationally accepted histopathological classification.

In North America the classification proposed at the Centennial Conference on Laryngeal Cancer[1] is usually used: keratosis, keratosis with atypia, carcinoma in situ. Others have also based their classifications on the degree of dysplasia[2-6], giving three groups: group 1 = hyperplasia or keratosis or both, with or without mild dysplasia; group 2 = moderate dysplasia; and group 3 = severe dysplasia and carcinoma

in situ. In Europe a classification introduced by Kleinsasser is often used[7]. This classification consists of three classes: class I = simple squamous cell hyperplasia; class II = squamous cell hyperplasia with atypia; class III = carcinoma in situ. Although the aforementioned classifications use a different nomenclature, they all distinguish three categories and, in fact, these categories more or less coincide. In the Netherlands Kleinsasser's classification has traditionally been used.

Kleinsasser[8], Delemarre[9], and Lubsen[10] have all shown by means of retrospective clinical pathological analyses that Kleinsasser's classification has prognostic significance: the risk of future development of carcinoma increases from class I to class III. We therefore base our management policy on this classification. In all cases microsurgical removal of the entire lesion is attempted. When classes I or II are reported by the pathologist no further treatment is given and the patient is merely followed up. With class III lesions, depending on the age of the patient and the site and extent of the lesion, further treatment, consisting of either CO_2 laser surgery or radiotherapy, is carried out.

Kleinsasser's classification, however, is subjective. In practice the problems encountered in the use of this classification focus particularly on the differential diagnosis between class II and III. Even between experienced pathologists in this field there is disagreement in diagnosis of a substantial number of cases. Furthermore, on review of the section, the classification is not always reproducible. It is therefore important to search for objective parameters which distinguish the different classes to improve the reproducibility of the grading of the hyperplastic laryngeal lesions so that optimal treatment of these lesions may be given. This study attempts to determine whether quantitative morphological analysis of the epithelial characteristics can contribute to the histopathological classification of squamous cell hyperplasia of the larynx.

MATERIAL AND METHODS

Material

A total of 50 microscope slides were examined. Forty five slides (15 each of class I, class II, and class III) were selected by two experienced, independent pathologists from a total of 275 slides pertaining to the patient material (total number of patients 103) of the Department of Otolaryngology, Free University Hospital, and the Netherlands Cancer Institute analysed earlier by Lubsen. These 45 slides were chosen at random, without knowledge of the patients' histories. Five slides from normal laryngeal epithelium were also included. All patients had been followed for at least six years.

Histology

All laryngeal specimens were fixed in formalin and embedded in paraplast (4 μm thick). The sections were stained with haematoxylin and eosin. Measurements for this study were carried out in three layers of the epithelium: the basal cell layer, consisting of one layer of basal cells; the prickle cell or intermediate cell layer; and the superficial cell layer. These layers are defined as follows: the basal cell layer is represented by a row of cells following the basal cell lamina; the superficial cell layer (or granular cell layer) is restricted to a superficial layer of three to five cells (below the stratum corneum when present) and characterised by densely distributed keratohyalin granules. In between these two strata is the remaining intermediate layer or stratum spinosum[11]. Measurements in this layer are made in the middle of the epithelial width (measured from the basement membrane to the upper layer of the stratum granulosum).

Definition of the histological classes

According to Kleinsasser's classification[7 8], with modifications of Delemarre[9], in class I, simple squamous cell hyperplasia, there is a thickening of the epithelium and often a form of keratosis (Fig. 1b; Fig. 1a shows normal epithelium). There is, however, no atypia. Mitoses are rare and found only in the basal cell layer. Class II, squamous cell hyperplasia with atypia (Fig. 1c) shows all the characteristics of the former and, in addition, atypia. A moderate number of mitoses are seen, some of which might be atypical. The atypia does not involve the full height of the epithelium. Class III, carcinoma in situ (Fig. 1d), manifests the generally accepted characteristics of carcinoma with the exception of invasive growth. There is a full thickness epithelial replacement by atypical cells. Mitoses are frequent and not limited to the basal cell layer.

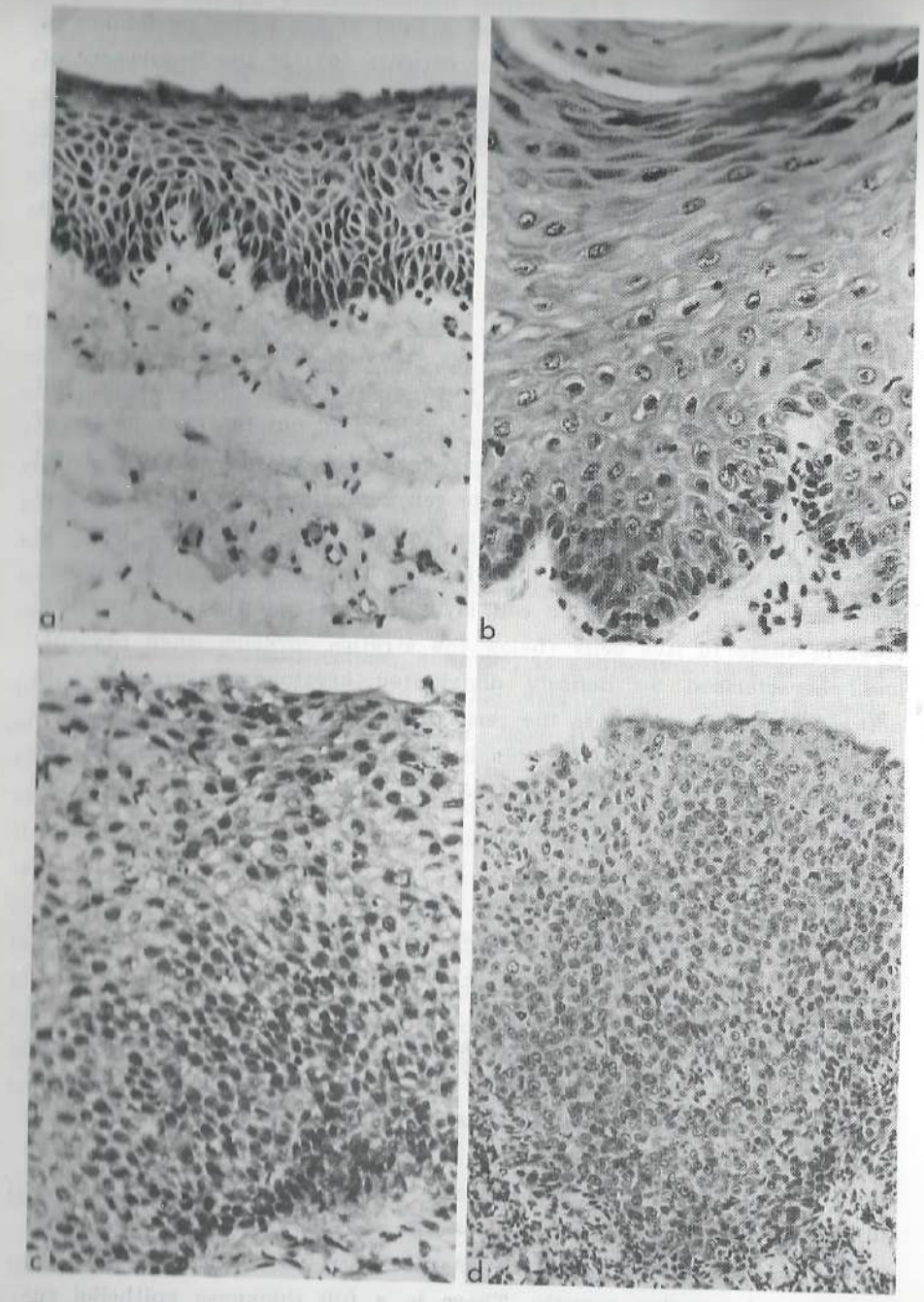

Fig. 1 (a) Normal squamous cell epithelium (x 330). (b) Simple squamous cell hyperplasia (x 330). (c) Squamous cell hyperplasia with atypia (x 330). (d) Carcinoma in situ (x 208).

Morphometry and statistical analysis

From each slide the most characteristic part of the lesion was selected for measurement by two experienced pathologists. Because the pathologist included the three layers of the epithelium in his evaluation of the lesion, 50 nuclei per cell layer were measured at random. The nuclear area, the nuclear contour index, and the polarity of these 50 cells were measured with a projection microscope (100x). The slides were projected on to a graphic digitising tablet (Bit Pad One, Summagraphics Corporation) (Fig. 2). Additional measurements were made of the nuclear crowding and the epithelial width. With these parameters the characteristics of the classification (hyperplasia and atypia) were assessed. There were no differences of any significance between measurements done twice by the same person at different places within the areas selected by the pathologists (Table 1). The data were transferred to a computer (Cyber 750, Control Data). The mean values, standard deviations, and correlations were calculated (Statistical Package for Social Sciences). For statistical analysis, Wilcoxon's non-parametric two sample test was used. We also attempted to measure the cell size but the quality of these sections fixed in formalin did not allow for accurate measurement of the cell size.

Fig. 2 Technical equipment: At the centre the digitising tablet on which the microscope image is projected by a mirror. On the right the standard computer terminal on which the results are shown. The average time to measure 50 cells is 15 min.

Table I: Examples of the mean nuclear areas, the mean nuclear contour indices and the mean nuclear polarities of 50 cells of the intermediate layer of 3 cases (1 class I, 1 class II and 1 class III), measured twice at different places within the same area by the same person.

	Measurement	class I	class II	class III
Mean nuclear area (μm^2)	1	71.04	103.23	90.90
intermediate layer	2	72.28	101.98	92.11
Mean nuclear contour index	1	3.64	3.61	3.69
intermediate layer	2	3.69	3.63	3.72
Mean nuclear polarity (radians)	1	0.74	1.31	1.71
intermediate layer	2	0.70	1.35	1.76

The following morphometric parameters, all carried out in the three cell layers, with exception of no 5 and no 6, were used to compare the three classes of Kleinsasser's classification:

1 The mean nuclear area (in μm^2).

2 The mean nuclear contour index (NCI), as given by the formula

$$NCI = \frac{P}{\sqrt{A}},$$

where P denotes the perimeter of the nucleus and A the nuclear area. The NCI is a size independent shape parameter. Its minimal value is found for a circle and is 3.545

$$\left(\frac{2\pi R}{\sqrt{\pi R^2}} = 2\sqrt{\pi} = 3.545 \right).$$

It increases when the nuclear irregularity increases[12].

3 The mean polarity of the nuclei, a parameter for nuclear axis orientation. The angle between the long axis of the nuclei and the local basement membrane was used as a measure of the polarity of the nuclei. The direction of the long axis was estimated by the computer from the coordinates of the perimeter points, obtained during the measurement of the nuclear area. This results in a mean nuclear polarity of nuclei in the basal cell layer (when the axes are generally orientated perpendicular to the local basement membrane) of ± 1.57 radians (360°= 2 π radians = 6.2830 radians; 1 radian ≈ 60°) and in a mean nuclear polarity of the nuclei in the superficial layer (when the axes are generally orientated parallel to the surface) of ± 0.0 radians.

4 The total number of nuclei per (arbitrary) square unit. The number of nuclei per square unit in the specimen is a measure of nuclear crowding (number of nuclei per unit volume)[13].

5 The maximum width of the epithelium, a measure of hyperplasia (arbitrary units). Strictly speaking, only a plane that is cut perpendicular to the epithelial surface (and if possible also perpendicular to the basement membrane) can serve for a proper measurement of the epithelial thickness. Only those parts of the sections which were vertical to the epithelial surface were selected for the measurement. No matter what method is applied this parameter remains somewhat unreliable.

6 The maximum width of the stratum corneum, a measure for (hyper-) keratosis.

7 The standard deviation (SD) for the various nuclear areas, nuclear contour indices, and nuclear polarities: SD nuclear area may be considered a measure of anisokaryose; SD nuclear contour index a measure of polymorphia; and SD nuclear polarity a measure of irregularity or architectural disturbance of the corresponding epithelial layer.

RESULTS

Table 2 gives the results of the measurements of the parameters of the normal epithelium and the three classes (I, II, III) of abnormal epithelium. The results of the statistical analysis carried out to compare the various classes are recorded in Table 3.

There was a significant difference between normal epithelium and class I, class I and class II, class I and class III for at least five of the parameters used (and even more when the standard deviations were taken into account), but between class II and class III significant differences were found for only three parameters. In Fig. 3 the mean nuclear polarity of the intermediate layer has been plotted against the nuclear crowding in the superficial layer of all classes (normal, classes I, II, and III). These two parameters were the most discriminating for

Table 2. Descriptive statistics; the mean values and standard deviations of the measurements of normal squamous cell epithelium and of the three classes of squamous cell hyperplasia (I, II, III).

	Normal	Class I	Class II	Class III
Mean epithelium width (au)	0.23± 0.11	0.51± 0.26	0.58± 0.29	0.43± 0.23
Mean width stratum corneum (au)	-	0.17± 0.32	0.16± 0.22	0.07± 0.06
Basal cell layer				
Mean nuclear area (μm^2)	56.28± 5.9	52.72±13.7	76.98±28.6	72.62±26.8
Mean nuclear contour index	3.81± 0.23	3.73± 0.18	3.82± 0.14	3.82± 0.12
Mean nuclear polarity (radians)	1.27± 0.13	1.33± 0.12	1.46± 0.16	1.67± 0.18
Mean nuclear crowding (au)	50.4 ± 6.2	46.4 ±13.7	32.1 ±10.4	38.8 ±12.1
Intermediate cell layer				
Mean nuclear area (μm^2)	91.01±14.8	72.23±15.1	108.29±33.1	95.68±22.9
Mean nuclear contour index	3.69± 0.05	3.64± 0.09	3.66± 0.07	3.7 ± 0.1
Mean nuclear polarity (radians)	0.38± 0.22	0.73± 0.31	1.21± 0.52	1.78± 0.33
Mean nuclear crowding (au)	34.4 ± 2.6	25.6 ± 5.0	24.8 ± 8.8	30.2 ±10.3
Superficial cell layer				
Mean nuclear area (μm^2)	55.2 ±14.6	59.9 ±13.1	83.9 ±39.5	66.0 ±14.2
Mean nuclear contour index	4.18± 0.23	3.80± 0.11	3.84± 0.17	3.87± 0.13
Mean nuclear polarity (radians)	0.21± 0.1	0.27± 0.12	1.13± 0.74	1.64± 0.68
Mean nuclear crowding (au)	21.6 ± 4.7	17.2 ± 5.3	15.0 ± 4.8	25.3 ± 8.7

au = arbitrary units.

Table 3. Differences between the classes, using Wilcoxon's two sample test; P denotes the level of significance (class I, II and III, and normal epithelium).

	Class I-Normal	Classes I-II	Classes I-III	Classes II-III
Mean epithelium width	$p < 0.05$	-	-	$p = 0.07$
Mean width stratum corneum	$p < 0.05$	-	-	-
Basal cell layer				
Mean nuclear area	-	$p < 0.01$	$p < 0.05$	-
Mean nuclear contour index	-	-	-	-
Mean nuclear polarity	-	$p < 0.05$	$p < 0.01$	$p < 0.01$
SD nuclear area	-	$p < 0.01$	$p < 0.01$	-
SD nuclear contour index	-	-	-	-
SD nuclear polarity	-	-	$p < 0.01$	-
Mean nuclear crowding	-	$p < 0.01$	-	-
Intermediate cell layer				
Mean nuclear area	-	$p < 0.01$	$p < 0.01$	-
Mean nuclear contour index	-	-	-	-
Mean nuclear polarity	$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$
SD nuclear area	$p = 0.07$	$p < 0.01$	$p < 0.01$	-
SD nuclear contour index	-	$p < 0.01$	-	-
SD nuclear polarity	-	$p < 0.01$	$p < 0.01$	-
Mean nuclear crowding	$p < 0.01$	-	-	-
Superficial cell layer				
Mean nuclear area	-	-	-	-
Mean nuclear contour index	$p < 0.01$	-	-	-
Mean nuclear polarity	-	$p < 0.01$	$p < 0.01$	$p = 0.06$
SD nuclear area	-	$p < 0.05$	-	-
SD nuclear contour index	-	-	-	-
SD nuclear polarity	-	$p < 0.01$	$p < 0.01$	-
Mean nuclear crowding	-	-	$p < 0.01$	$p < 0.01$

SD = standard deviation.

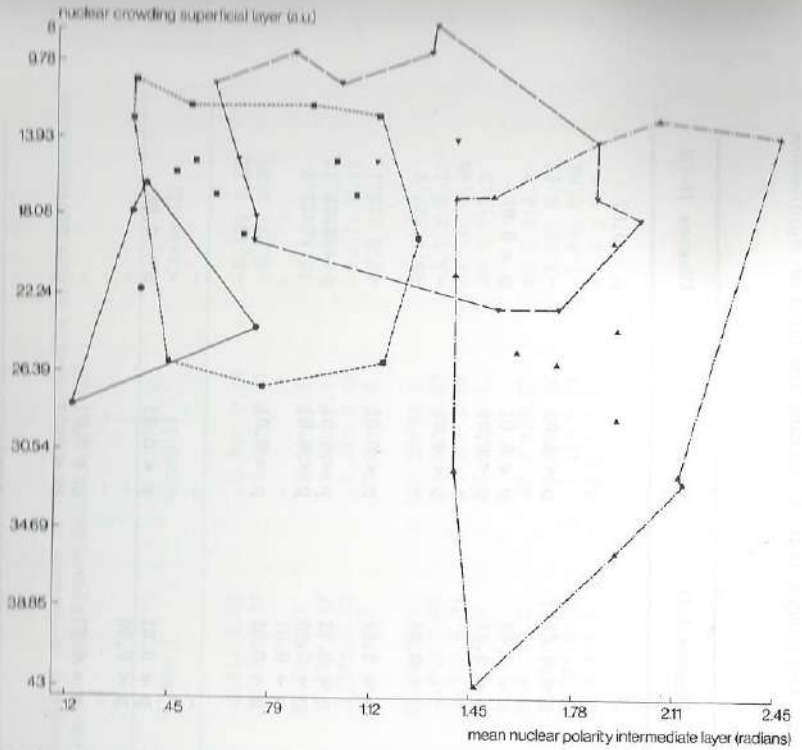

Fig. 3 Scattergram of all cases: normal epithelium ●, class I ■, class II ▼, class III ▲.

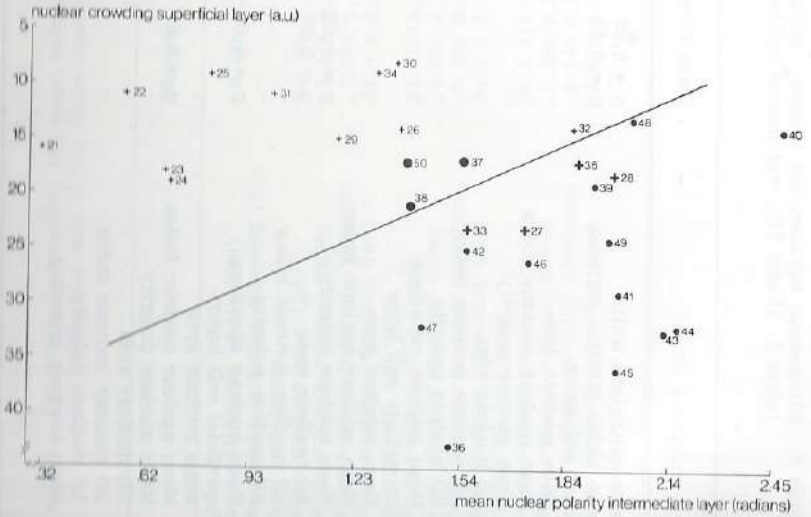

Fig. 4 Scattergram of all cases of class II and class III; + class II (patients 21-35) and ● class III (patients 36-50).

class II and class III, according to a stepwise discriminant analysis[14]. From a graphic representation (Fig. 4) with a function line, which denotes the discriminant function, it appears that within the group of lesions classified as class II one cluster (patients 27, 28, 33, and 35) more or less coincides with class III. Three patients of class III (patients 37, 38 and 50) are above the function line, close to the group of patients of class II. The function line drawn to give an optimal separation of class II and class III has been established by means of a function classification, using the following function: 1.489 x Polar. I. + 0.1047 x NUC.S - 4.35 = 0.*

A retrospective analysis of the records of patients classified as class II has been carried out to see whether lesions from patients above the line, if not treated other than by endoscopic biopsy, run a different course from those below the line. Seven of 15 patients were excluded from the comparison for the following reasons. Three patients (26, 33, and 34) at the time had been treated with radiotherapy for their class II lesion. If an infiltrating carcinoma was diagnosed within one year of the first biopsy, as was the case in two patients (21 and 23), those patients were excluded as well on the assumption that the first biopsy was probably not representative of the lesion. There was also doubt about the first biopsy of one patient (22); within some months after the first biopsy (class II) a second biopsy was done, in which a class III lesion was found. Finally, one patient (29) was excluded because he had been treated with radiotherapy nine years before for an unknown condition of the larynx. Of the remaining five patients of class II above the discriminant function line, one (31) developed a carcinoma after eight years and another (25) showed a carcinoma in situ (class III) within two and a half years. The other three patients of this group followed a favourable clinical course. Of the two patients of the group of class II left below the discriminant function line, one (27) developed a carcinoma after 10 years, whereas another (35) had developed a carcinoma in situ after one year.

All patients of class III have been treated, either with radiotherapy (n=13) or they underwent a cordectomy (n=2). Thus long term follow up on non-treated patients within this group is impossible.

* Polar. I. = mean nuclear polarity intermediate layer; NUC.S. = mean nuclear crowding superficial layer.

DISCUSSION

The histological classification of squamous cell lesions is very subjective.[5] Few studies applying objective methods have been carried out to assess the epithelial characteristics of the so called premalignant lesions of the laryngeal mucosa.

Several workers[2-4 15 16] have performed photometric studies determining nuclear DNA content and nuclear area. Hellquist et al[3] have investigated, in particular, patients with lesions with dysplasia; the nuclei measured in their study were taken at random through the epithelium. Their findings did not show any morphological or photometric differences between the epithelia with moderate dysplasia which subsequently developed severe dysplasia or carcinoma in situ and those that did not. Apart from doubt regarding its usefulness, it appears unlikely that spectrophotometric analysis will be used routinely if only because it cannot be performed on routine stained and one also needs expensive photometric equipment.

Morphometry using simple objective parameters which can be applied on any routine processed material has proved to be of use in grading all kinds of tumours, including bladder[17] and prostate tumours.[18] In this study morphometry was applied to histological slides from patients with squamous cell hyperplasia of the laryngeal epithelium, classified according to Kleinsasser.[7]

Measurements of the nuclei were done in the three layers of the epithelia that can be distinguished: basal, intermediate, and superficial. This appears to be an essential difference from the approach of Hellquist et al. With morphometric parameters we showed that between class I and class II and between class I and class III there are six siginificant parameters. Thus with the assistance of morphometry, especially with the mean nuclear area of the basal and intermediate cell layer (an increase of the nuclear area from class I to class II and from class I to class III in both layers) and with the mean nuclear polarity in all three layers (the polarity expressed in radians increases from class I to class II and from class I to class III in the three layers each) one can easily distinguish between these classes (class I and class II, and class I and class III).

The differentiation between class II and class III, however, is more difficult. Only three significant morphometric parameters between

these classes were found. Application of a stepwise discriminant analysis (the features are arranged in a certain sequence to discriminate between the groups; with a combination of two parameters: the mean nuclear polarity of the intermediate layer and the mean nuclear crowding of the superficial layer (Fig. 4)) results in a division into two groups that is not indentical to the division by histopathological examination alone: a small cluster of histologically classified class II lesions below the discriminant function line morphometrically falls into the class III category. Unfortunately, we could not determine whether this particular morphometrically identified cluster of class II lesions followed a clinical course typical for class III lesions (for several reasons many patients had to be excluded in the retrospective clinical analysis).

Histological revision of lesions, particularly of class II and class III, even by experienced pathologists is not reproducible in many cases. In this study nuclear areas, shape factors, crowding, and epithelial width have been measured. The morphometric findings are highly reproducible for the classification of squamous cell hyperplasia of the laryngeal mucosa. Furthermore, the findings suggest that the usual histopathological classification of Kleinsasser may be inadequate for there is evidence of two groups within class II above and below the discriminant function line. The clinical course of these two groups needs study and definition.

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THE CLINICAL RELEVANCE OF CLASSIFICATION OF SQUAMOUS CELL HYPERPLASIA OF THE LARYNX BY MORPHOMETRY.

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SUMMARY

By means of morphometry, differentiation between the classes of laryngeal squamous cell hyperplasia can easily be performed, mutually (Kleinsasser's classification) and in comparison with normal epithelium. It is rather difficult, however, to distinguish between class II (hyperplasia and atypia) and class III (carcinoma in situ). In the group of class II lesions, two groups of patients could be differentiated a prognostically favourable and unfavourable group. When using a linear discriminant analysis, the two groups mentioned could be distinguished morphometrically. In histopathological examination, lesions classified as hyperplasia and atypia (class II), must also be examined with morphometry, because in this way the group at risk can be traced.

INTRODUCTION

In grading the 'premalignant' lesions which develop from the squamous epithelium of the larynx, hyperplasia and atypia are the essential features. Therefore Kleinsasser tried to resolve the confusion about the terminology and the several descriptions of hyperplastic lesions of the laryngeal epithelium and introduced the term squamous cell hyperplasia[1,2]. During recent decades many cases of transition from hyperplastic and similar lesions into invasive carcinoma have been described. These studies showed that, in analogy with cervical dysplasia, there is

a continuous ranging from hyperplastic lesions to carcinoma in situ and that subsequently an invasive carcinoma can develop.

Comparison of the various studies is not easy because of the variety of histopathological classification and terminologies used by different authors. In retrospective clinical analyses, Kleinsasser's

Table I. The numbers of non-treated class I, class II and class III patients, who developed an invasive carcinoma

	Class I	Class II	Class III	Mean interval
Kleinsasser (4), 1963	5/61	1/5	18/20	6 yr
Delemarre (3), 1970	3/20	6/26	4/8	2½ yr
Lubsen (5), 1980	1/23	5/18	1/3	5½ yr

classification has proved of interest[3 4 5] (Table 1). Hyperplastic changes in the epithelium of the larynx fall into one of the three classes according to the degree of cell atypia present: class I: simple squamous cell hyperplasia; class II: squamous cell hyperplasia with atypia; class III: carcinoma in situ. However, it is impossible to actually draw a sharp line between class II and class III and the differential diagnosis as to squamous cell carcinoma may present problems. There will always be a subjective element in the diagnosis of a pathologist, which is why objective parameters may be useful.

Several objective methods such as photometry and electronmicroscopy have been adopted by various investigators to assess the epithelial characteristics of 'premalignant' lesions of for instance the oral mucosa[6 7 8] and of the laryngeal mucosa[9]. In an earlier study we have defined morphometrical parameters to differentiate the three classes[10]. The findings suggest that within class II, two groups with differing prognoses could be distinguished. Unfortunately, as most of the class II (and all class III) lesions were treated, this aspect could not be evaluated. Therefore in the present study only slides of non-treated patients were examined.

The objective of this study was to test the hypothesis on a large group of non-treated patients with long-term follow-up, classified on

the first taken biopsy as class II. The untreated patients from class I and class III will also be studied.

MATERIAL AND METHODS

Material

A total of 59 microscopical slides of non-treated patients (18 class I, 38 class II and 3 class III slides) and 10 controls with normal laryngeal epithelium, were selected by two of us (J.D., P.O.). The biopsy specimens were all first biopsies taken from patients with laryngeal hyperplasia from the files of the Department of Otolaryngology of the Free University Hospital and the Antoni van Leeuwenhoekhuis (The Netherlands Cancer Institute) both in Amsterdam. These patients were carefully followed up for a period of at least 6 years.

Histology

Formalin-fixed (10%) and Paraplast-embedded laryngeal specimens were used. The sections (4 μm thick) were stained with haematoxylin and eosin. The microscopical slides were reviewed and classified according to Kleinsasser's classification. The characteristic morphological features of the classes are, class I: broadening of the squamous epithelium, with a regular structure and without atypia; class II shows the characteristic morphological features of class I and besides cell atypia; the picture of class III is that of carcinoma (polymorphia and atypia) without invasive growth (Fig. 1, chapter III).

Morphometry

The degree of tissue organization is particularly relevant in morphometrical analysis. Anisotropic tissues demonstrate a layering or ordering into regular patterns and by definition, this occurs in stratified squamous epithelium; in isotropic tissues the components do not demonstrate any perferred orientation, the liver e.g.[11]. Individual cell layers within the epithelium form polarized sheets, within which the individual cells possess structural homogeneity[12 13]. The individual strata can be analyses[14] and the process of differentiation can be quantitatively assessed by comparing data obtained from successively higher epithelial layers[11]. In routine diagnosis pathologists classify the lesions according

to the histopathological features of the cells in the epithelial layers. Therefore we selected areas which showed the most characteristic features according to the class (this selection proved to be reproducible) and measured at random chosen nuclei in the three cell layers of the epithelium; the basal cell layer: a row of cells following the basal lamina; the superficial cell layer: restricted to a superficial layer of 3 to 5 cells, and characterized by densely distributed keratohyalin granules. In between these two layers is the intermediate layer of stratum spinosum[6]. Measurements in this layer are carried out in the middle of the epithelial width. The slides were projected with a projection microscope (100x) on a digitising tablet (Summagraphics Corp.). Of each layer, 50 nuclei were measured. The nuclear crowding was estimated from photographs taken from the microscopical slides (enlargement 100x) within a fixed, but arbitrary square. The data were transferred to a PDP 11/40 (Digital Equipment Corp., Maynard, Mass.) and after simple transformation, transferred further to a Cyber 750 (Control Data) for statistical analysis.

From each case measured, the following parameters were calculated in each of the three cell layers, with the exception of (e) and (f):

(a) Mean nuclear area (μm^2); its standard deviation is a measure of anisokaryose.

(b) Mean nuclear contour index; the NCI is a size-independent shape parameter, defined as $\frac{\text{Perimeter}}{\sqrt{\text{area}}}$

Its minimal value is found for a perfect circle and is 3.545.

$$\left(\frac{2\pi R}{\sqrt{\pi R^2}} = 2\sqrt{\pi} = 3{\cdot}545\right).$$

The NCI increases when the nuclear irregularity increases. Its standard deviation is a measure of polymorphia.

(c) Mean nuclear polarity (radians; $1 \approx$ radian 60°), a parameter for orientation of the long nuclear axis, for which the angle between the long axis of the nuclei and the local basement membrane was measured. When the axes are generally orientated perpendicular to the (local) basement membrane, as in the nuclei in the basal cell layers, a polarity of ± 1.57 radians is found. When the axes are generally orientated parallel to the surface, as in the nuclei in the superficial layer, a polarity of ± 0.0 radians is measured.

(d) Nuclear crowding (number of nuclei per unit volume[14]), the number of nuclei per arbitrary unit area.

(e) The epithelial width (Arbitrary Units, A.U.), a measure for hyperplasia; only those parts of the section which were vertical to the epithelial surface, were selected for the measurement.

(f) The width of the stratum corneum (A.U.) a measure for (hyper-) keratosis.

An attempt has been made to measure the cell size as well, however, the quality of these sections fixed in formalin did not allow for accurate measurements of the cell size.

Statistical analysis

Descriptive statistics were calculated for each class respectively. Wilcoxon's test was used to establish the difference between the classes. As the level of significance, $p < 0.05$ was adopted. A multivariable analysis was performed and the most discriminating parameters (stepwise discriminant analysis; method: RAO's V[15]) were used to distinguish between the group of patients from class II that developed invasive carcinoma or became a carcinoma in situ (class III) and the group of patients that did not.

RESULTS

The descriptive statistics calculated from the morphometrically assessed features of the nuclei and of the epithelial width, of the normal epithelium and the three classes (I, II and III) are recorded in Table II. In Table III the significant parameters between the classes are listed. When comparing the control group with normal laryngeal epithelium, class I and class III, many significant parameters were found. Class III consists of only 3 non-treated cases, and we therefore added the results of measurements of class III from an earlier study (10). As to class II and class III, the comparison is more difficult.

In a scattergram (Fig. 2) the mean nuclear contour index in the basal layer of all cases of class II is plotted against the mean nuclear crowding in the basal layer. The numbers correspond to the patient numbers in Table IV, in which the patients histologically classified as class II are also recorded according to their clinical follow-up.

Table II. Descriptive statistics; the mean values and standard deviations of the measurements of normal squamous cell epithelium and of the three classes of squamous cell hyperplasia (I, II, III). Class II is divided in group IIa and group IIb; see text.

	Normal	Class I	Class II	Group IIa	Group IIb	Class II
Mean epithelial width (A.U.)	0.11±0.04	0.48±0.19	0.38±0.24	0.42±0.29	0.33±0.16	0.39±0.22
Mean width stratum corneum (A.U.)	-	0.12±0.27	0.07±0.15	0.10±0.21	0.05±0.05	0.07±0.06
Basal cell layer						
Mean nuclear area (μm^2)	45.34±6:6	60.87±18.5	64.88±26.2	65.0 ±27.3	64.72±25.6	75.23±25.5
Mean nuclear contour index	3.73±0.14	3.82±0.15	3.84±0.15	3.81±0.14	3.87±0.17	3.81±0.11
Mean nuclear polarity (radians)	1.53±0.25	1.44±0.17	1.56±0.18	1.55±0.19	1.58±0.18	1.65±0.17
Mean nuclear crowding (A.U.)	56.0±2.3	40.9±11.4	40.1±10.6	38.7±10.4	41.8±10.9	38.3±11.0
Intermediate cell layer						
Mean nuclear area (μm^2)	62.47±9.2	83.51±15.2	83.92±31.7	80.79±30.0	87.79±34.2	96.19±21.3
Mean nuclear contour index	3.69±0.07	3.65±0.08	3.7±0.1	3.69±0.11	3.71±0.09	3.7±0.09
Mean nuclear polarity (radians)	1.42±0.25	1.28±0.6	1.52±0.36	1.52±0.44	1.51±0.25	1.74±0.33
Mean nuclear crowding (A.U.)	43.6±7.4	23.2±6.7	28.9±9.6	28.5±8.7	29.4±10.9	29.6±9.5
Superficial cell layer						
Mean nuclear area (μm^2)	38.0±5.3	60.2±16.4	80.26±36.5	78.21±42.2	82.79±29.2	69.25±15.8
Mean nuclear contour index	4.16±0.23	3.95±0.21	3.83±0.15	3.82±0.13	3.84±0.17	3.85±0.13
Mean nuclear polarity (radians)	1.58±0.44	1.15±0.9	1.49±0.65	1.40±0.71	1.59±0.56	1.63±0.65
Mean nuclear crowding (A.U.)	32.8±8.2	16.1±4.7	20.7±13.2	17.7±5.7	24.4±18.3	23.9±8.5

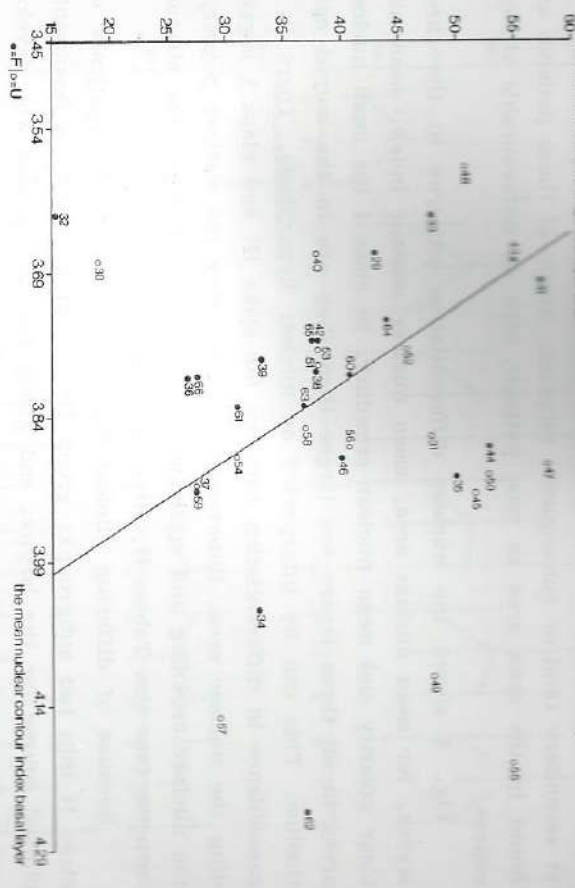

Fig. 2. All cases in class II. Most patients who subsequently developed an invasive laryngeal carcinoma, or developed a carcinoma in situ, are found to the right of the discriminant function line. ● Patients with a favourable clinical course; ○ patients with an unfavourable clinical course. Patient numbers correspond to the numbers in Table IV.

Most patients (nos. 31, 37, 45, 47, 49, 50, 52, 54, 55, 56, 57 and 58) who developed an invasive carcinoma after 1, up to almost 10 years from the first biopsy, are clustered in the scattergram right of the discriminant function line (function: Mean Nuclear Crowding basal layer + 128.57 x Mean Nuclear Contour Index basal layer - 529.29). On the left side of this line, 5 patients (nos. 30, 40, 48, 51 and 53)are found with an unfavourable clinical course for the larynx. Patient no. 30 developed a carcinoma in situ (class III) within 2 years after the first biopsy; this was classified as class II. The other 4 patients developed an invasive carcinoma after 1½ to 6 years.

Whereas the remaining patients in class II (n=21) had a favourable clinical course for the larynx, 3 of these (nos. 38, 39 and 46) died, but not within 3 years after the first and only biopsy (no. 38: cerebrovascular accident; no. 39: primary pancreatic carcinoma; no. 46: primary bronchial carcinoma). All patients with a favourable clinical

course continued to have classifications lower than or equal to class II at secondary (and/or subsequent) biopsies. Seven of these patients are found in the same area as most patients with an unfavourable clinical course.

Fig. 3 shows the nuclear differentiation patterns in the three layers, for mean nuclear area, mean nuclear contour index, mean nuclear polarity and mean nuclear crowding. In class I the mean nuclear areas in all three layers are larger than they are in the normal epithelium. This can be interpreted as nuclear hyperplasia. There is a resemblance in differentiation patterns for class III and class I regarding the nuclear area. However, when comparing the nuclear polarity, the nuclear crowding and epithelial width and the width of the stratum corneum (see also Tables II, III), there are differences.

Because of differing clinical courses we divided the patients of class II into two subgroups: group IIa: 21 patients with a favourable clinical course for the larynx, and group IIb: 17 patients who developed a laryngeal carcinoma or carcinoma in situ (class III). For this reason these patients received a full course of radiotherapy and in the case of a recurrence they were treated with a partial laryngectomy (nos. 37 and 48) or a total laryngectomy (nos. 31, 40, 49 and 53).

In group IIb the mean nuclear areas in the intermediate and superficial layer tended to be larger than the areas in the same layers of group IIa (Table II, Fig. 3). However, no statistically significant parameters were found for group IIa and group IIb (Table III).

When using a linear stepwise discriminant analysis (method: RAO's V^{15}) for groups IIa and IIb, after 5 steps a p-value of 0.0093 is found (steps 1-5: mean nuclear contour index basal layer, mean nuclear crowding basal and superficial layer, mean nuclear area superficial layer and standard deviation mean nuclear area basal layer). The function (F) for discrimination of the groups is: 4.71 x mean nuclear contour index basal layer +0.01 x mean nuclear area superficial layer +0.23 x standard deviation mean nuclear area basal layer +0.16 x mean nuclear crowding basal layer +0.06 x mean nuclear crowding superficial layer -31.25; where F≥0 denotes: unfavourable prognosis and F<0: favourable prognosis.

When the five steps are included in the analysis, 79% of the individual cases of groups IIa and IIb can be correctly classified with an overall probability of 0.82 (range: 0.62-0.97) (Table IV), if a decision

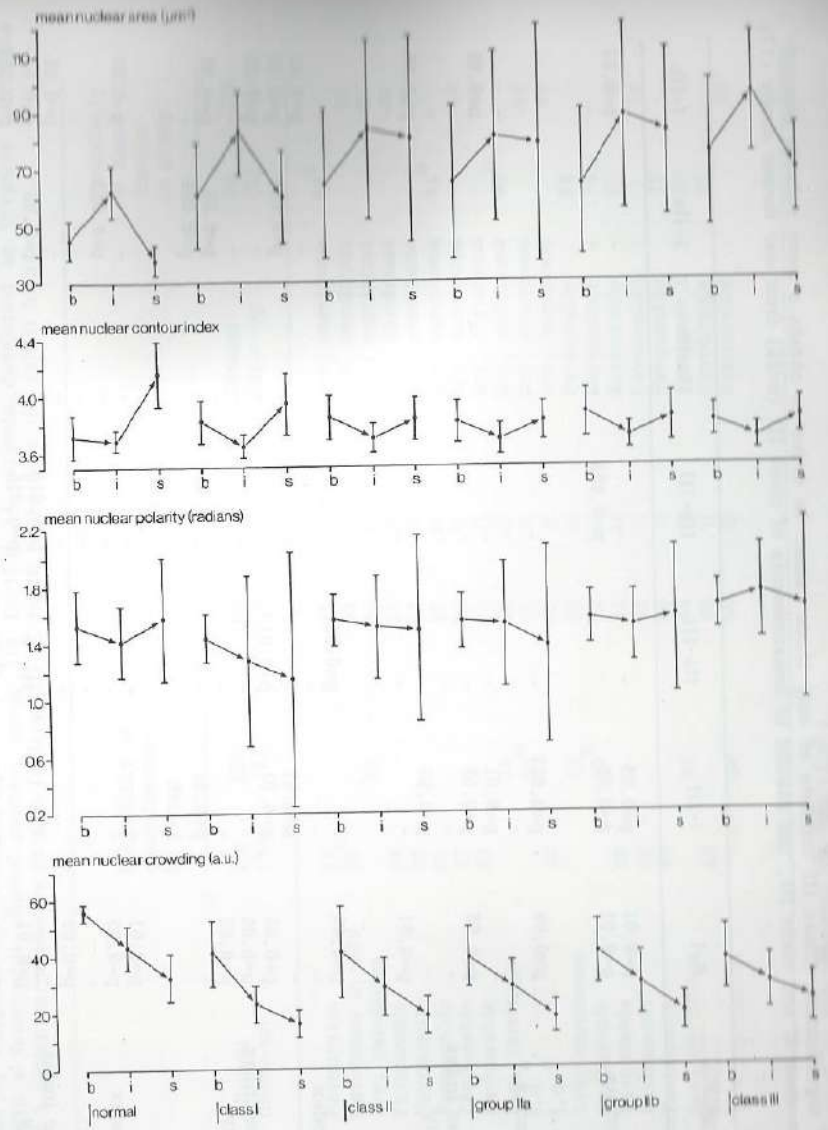

Fig. 3. Nuclear differentiation patterns (mean values and standard deviations of the mean nuclear area, the mean nuclear contour index, the mean nuclear polarity and the mean nuclear crowding) in the three layers (b=basal cell layer, i=intermediate cell layer, s=superficial cell layer) of epithelia with squamous cell hyperplasia.

Table III. Differences between the classes (I, II, III and the groups of class II: group IIa and group IIb) and normal epithelium (N), using Wilcoxon's two-smaple test.

p denotes the level of significance. Class III consists of only three cases. In order to obtain a more reliable comparison, especially between class II and class III, the results of measurements of class III (n=15) from our former study (13) have been added.

	N-I	I-III	IIa-III	IIb-III	IIa-IIb	1-IIa	I-IIb
Mean epithelial width	p<0.01	p<0.05	-	-	-	-	p<0.01
Mean width stratum corneum	p<0.01	p<0.05	-	p=0.052	-	-	-
Basal cell layer							
Mean nuclear area	p<0.05	p=0.057	-	-	-	-	-
Mean nuclear contour index	-	-	-	-	-	-	-
Mean nuclear polarity	-	p<0.01	-	-	-	-	p<0.05
SD mean nuclear area	p<0.05	p<0.05	-	-	-	-	-
SD mean nuclear contour index	-	-	-	-	-	-	-
SD mean nuclear polarity	-	p<0.05	-	-	-	-	p<0.05
Mean nuclear crowding	p<0.01	-	-	-	-	-	-
Intermediate cell layer							
Mean nuclear area	p<0.01	-	p<0.05	-	-	-	-
Mean nuclear contour index	-	-	-	-	-	-	p<0.05
Mean nuclear polarity	-	p<0.01	-	p<0.05	-	p=0.052	p<0.05
SD mean nuclear area	p<0.05	p<0.01	p<0.01	-	-	-	p<0.05
SD mean nuclear contour index	p<0.05	-	-	-	-	-	-
SD mean nuclear polarity	p<0.05	-	-	-	-	p<0.05	p<0.05
Mean nuclear crowding	-	p<0.05	-	-	-	p=0.055	-
Superficial cell layer							
Mean nuclear area	p<0.01	-	-	-	-	-	p<0.05
Mean nuclear contour index	p<0.05	-	-	-	-	p=0.059	-
Mean nuclear polarity	-	-	-	-	-	-	-
SD mean nuclear area	p<0.05	-	-	-	-	-	p<0.05
SD mean nuclear contour index	-	-	-	p=0.056	-	p<0.05	p<0.01
SD mean nuclear polarity	p<0.01	-	-	p=0.055	-	-	p=0.059
Mean nuclear crowding	-	p<0.01	p<0.05	-	-	-	p<0.05

Table IV. Classification by morphometry (using 5 parameters) in 38 patients, histologically classified in class II; group IIa (n=21) patients with a favourable clinical course; group IIb (n=17), 16 patients developed an invasive carcinoma (=inv. carcinoma) and 1 patient a carcinoma in situ (=class III), 1 to 10 years from the initial biopsy.

Pat. no.	Histological classification	Clinical follow-up	Probability of classification in group IIa (%)	Probability of classification in group IIb (%)	Pat. no.	Histological classification	Clinical follow-up	Probability of classification in group IIa (%)	Probability of classification in group IIb (%)
29	II	Favourable	74		48	II	Inv. carcinoma	80[b]	
30	II	Carcinoma in situ	60[b]		49	II	Inv. carcinoma		63
31	II	Inv. carcinoma		81	50	II	Inv. carcinoma		97
32	II	Favourable	84		51	II	Inv. carcinoma		96
33	II	Favourable	90		52	II	Inv. carcinoma		97
34	II	Favourable	80		53	II	Inv. carcinoma	91[b]	
35	II	Favourable	67		54	II	Inv. carcinoma		69
36	II	Favourable	69		55	II	Inv. carcinoma		90
37	II	Inv.[a] carcinoma		70[b]	56	II	Inv. carcinoma	89[b]	
38	II	I.d.[a]		55[b]	57	II	Inv. carcinoma		84
39	II	I.d.[a]	90		58	II	Inv. carcinoma		89
40	II	Inv. carcinoma		62	59	II	Favourable	80	
41	II	Favourable		65[b]	60	II	Favourable	74	
42	II	Favourable	68		61	II	Favourable	97	
43	II	Favourable	82		62	II	Favourable		54
44	II	Favourable	89		63	II	Favourable	73	
45	II	Inv.[a] carcinoma		89	64	II	Favourab:e	90	
46	II	I.d.[a]	89		65	II	Favourable	67	
47	II	Inv. carcinoma		94	66	II	Favourable		86[b]

[a] Three patients died from an intercurrent disease (I.d.).
[b] Misclassified.

threshold to distinguish between the groups of 0.50 is taken. When a threshold of 0.80 is adopted, 29% of the cases fall in a 'doubtful' group. The figure of 79% is optimistic when the decision rule is applied for general diagnosis, because for evaluation of the rule the same population was used as for assessing this decision rule.

Looking back to the scattergram (Fig. 2) 5 patients with an unfavourable clinical course for the larynx were found left of the discriminant function line in the middle of most cases with a favourable clinical course (group IIa). Three of these patients were - although they had an unfavourable clinical course - according to the morphometrical classification, classified in group IIa (nos. 30, 48 and 53, with a probability of 0.60, 0.80 and 0.91 respectively). Of the remaining 2 patients, no. 40 was morphometrically classified in group IIb with a probability of just 0.62 ('doubtful' case) and no. 51 with 0.96. So in this scattergram the only incorrectly graphically reflected but morphometrically correctly classified patient was no. 51.

DISCUSSION

For a consistent grading of 'pre-cancerous' squamous cell lesions of the laryngeal epithelium, quantitation of characteristics of hyperplasia and atypia may be helpful. The basis of any quantitative study should be the approach of the pathologist towards routine diagnosis, which means that those cells should be quantitated on which his diagnosis is based. If such an approach to quantitation is maintained, the results obtained can be 'translated back' to routine practice[16]. Morphometry might reveal discriminant parameters that are not easily estimated by pathologists. Quantitation has already proved to be of use in, for instance, the grading of endometrial carcinoma[17] and the grading of (uterine) cervical lesions[18].

In a previous study[10] it could be demonstrated that one can easily distinguish between class I and class II, and between class I and class III. The differentiation between class II and class III was more difficult. Class II appeared not to be a clearly defined group. For that study, selection of the slides was made on their quality and on their representativity for the class in which the lesion was classified. In the present study, only non-treated patients were selected, which means no

treatment other than merely follow-up after endoscopic biopsy.

Between class I and the normal epithelium many significant morphometrical parameters are found. Class III consists of only three non-treated cases, therefore we added the results from the earlier study to make the comparison with for instance class I more reliable. However, in routine histopathological practice, differentiation between class I and class III as well as between class I and normal epithelium is not too difficult.

The patients in class II have been divided into 2 groups because of their differing clinical course (Tabel IV). Most of the patients (n=12) who finally developed a carcinoma (n=17), form in a scattergram (Fig. 2) a cluster in the right-hand section (group IIb). On the other hand, the group with - on the average - a more favourable clinical course is clustered in the left-hand section (group IIa). This scattergram was made from all cases of class II with use of parameters (the mean nuclear contour index basal layer and the mean nuclear crowding basal layer) which proved to be most discriminant for group IIa and IIb (stepwise discriminant analysis[15]).

In the study of differences between the groups in class II, none of the morphometrical parameters used reached statistical significance. When linear discriminant analysis was applied to the morphometrical parameters, clear differentiation between the groups in IIa and IIb was easy (by using five parameters, 79% of the cases in the two groups could be correctly classified, with an average probability of 0.82), while by light microscopical examiniation this differentiation was difficult. In the morphometrical discrimination between the prognostically favourable and unfavourable lesions, attention was paid to the nulear area in the superficial layer, the nuclear crowding in the basal and superficial layer and the nuclear polymorphism in the basal layer (nuclear contour index), all of which were found to be increased in the case of lesions from the prognostically unfavourable group (group IIb) vis-à-vis group IIa. By contrast, the fifth parameter included in the analysis, the nuclear anisokaryose in the basal layer (as expressed in the standard deviation mean nuclear area), proved to be less pronounced for the group prognostically unfavourably classified, when compared with the favourably classified group.

Only 3 of the patients (n=16) who were, according to the initial biopsy taken from the vocal cords, classified as class II (hyperplasia

and atypia) and who subsequently were found to have larynxcarcinoma, passed through the carcinoma in situ stage. The patients classified in the prognostically unfavourable group form a special group at risk of developing an invasive carcinoma. They need extra attention in the histopathological examination. The lesions histopathologically classified as class II, must also be examined by means of morphometry to establish a more accurate classification.

Further studies are being carried out on a test set, to evaluate the results of the decision rule as employed in this study.

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THE PROGNOSTIC SIGNIFICANCE OF MORPHOMETRY FOR SQUAMOUS CELL HYPERPLASIA OF THE LARYNGEAL EPITHELIUM.

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ABSTRACT

Laryngeal biopsies of squamous cell hyperplasia with atypia were graded by means of morphometry, using 5 non-correlated nuclear parameters preselected with linear discriminant analysis, and were tested for their prognostic significance, in a follow-up study. Fifty-two biopsy specimens were obtained from 18 patients who were merely followed and if necessary underwent a repeated biopsy examination. The lesions of 10 patients progressed to invasive carcinoma. In 8 patients the second or further biopsies were again classified as squamous cell hyperplasia with atypia. For both groups the mean follow-up period was 7.2 years (range: 3½-11). In the progressive group 73% of the biopsies were morphometrically classified as prognostic unfavourable, whereas the clinical outcome on the basis of the last biopsy was correctly predicted for 6 out of the 10 cases (mean probability: 94%), the other 4 cases were "mis-classified", although for 3 cases with a low probability (mean: 60%). For a valuable morphometrical classification of lesions of individual patients, the results as presented still are insufficient. Morphometrical classifiaction probabilities may be improved when the number of untreated patients with squamous cell hyperplasia with atypia do increase.

INTRODUCTION

For the treatment of epithelial hyperplasia of the larynx an exact

histopathological diagnosis is required. In our hospitals we use a three grade classification: class I, simple squamous cell hyperplasia; class II, squamous cell hyperplasia with atypia; class III, carcinoma in situ. This classification carries prognostic significance, which was shown by Kleinsasser, who introduced this classification[1] [2] and by two others [3] [4]; all three studies included large series of patients. Our management policy is based on this classification implying in case of class I or class II, merely follow-up of the patient. In class III lesions usually radiotherapy was applied and in some cases endoscopic CO_2 laser surgery or only endoscopical removal.

The reproducibility and objectivity of the diagnosis in lesions which vary from normal to malignancy as a continuous spectrum, as is the case in laryngeal epithelial hyperplasia, can be improved by quantitative objective methods[5]. It was shown before[6] that quantitative objective parameters can easily be applied on routinely processed material, in order to get a more reliable assessment of nuclear atypia, particularly in case of hyperplasia with atypia (class II) and carcinoma in situ (class III). In practice, the problems encountered in the use of this classification are found in that part of the continuous spectrum of hyperplastic squamous cell lesions of the larynx. The clinical relevance of a computed morphometrical decision rule for these type of lesions was shown in a former study[7]. To evaluate the prognostic significance in untreated patients with laryngeal squamous cell hyperplasia with atypia, a study has been carried out on a set of repeated biopsy specimens from patients initially classified in class II with long term follow-up.

MATERIAL AND METHODS

Patients

A total number of 118 patients with squamous cell hyperplasia of the laryngeal epithelium were seen between 1974 and 1981 in the Department of Otolaryngology of the Free University Hospital and in the Antoni van Leeuwenhoekhuis (the Netherlands Cancer Institute), both in Amsterdam, The Netherlands. Thirty-two patients were at revision classified as carcinoma in situ, 30 patients received for this reason a full course of radiotherapy. For this morphometrical investigation only biopsy specimens from untreated patients (n=18) with repeated biopsy

examinations were selected, performed at least 6 months after the initial biopsy, which has histologically classified as hyperplasia with atypia. In this study 52 laryngeal biopsy specimens have been examined.

According to their clinical outcome the 18 patients were divided into 2 groups, group 1: 8 patients with a favourable clinical course and group 2: 10 patients with an unfavourable clinical course. All patients were males and were followed-up for at least 5 years. The mean age for group 1 was 56 years (range: 28-80) and for group 2: 61.5 years (range: 46-78). Between group 1 and group 2 no pregnant differences were found for this small group of patients as to their smoking habits or alcohol consumption, the duration of the hoarseness and the localisation of the lesions on the vocal cords.

All the follow-up biopsies (n=26) of the 8 patients with a favourable clinical outcome, who did not receive any kind of treatment, were again classified as class II (Fig. 1). One of these patients (no. 5) died from a myocardial infarction, another patient (no. 6) from a primary bronchial carcinoma, 8 and 6 months respectively from the last biopsy examination.

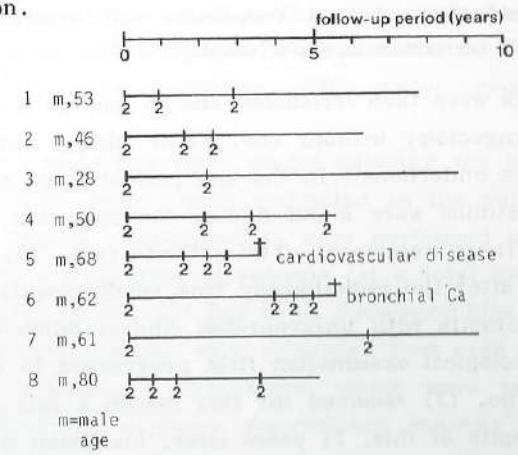

Fig. 1 Follow-up of 8 patients with an initial class II lesion (squamous cell hyperplasia and atypia).
On the further biopsies these patients were again classified as class II (=2) / † =died.

The 10 patients, who in their first biopsy showed squamous cell hyperplasia with atypia (class II) and had an unfavourable clinical course further on, were treated at the time their biopsy showed invasive laryngeal carcinoma (13 months - 7½ years after the first biopsy)

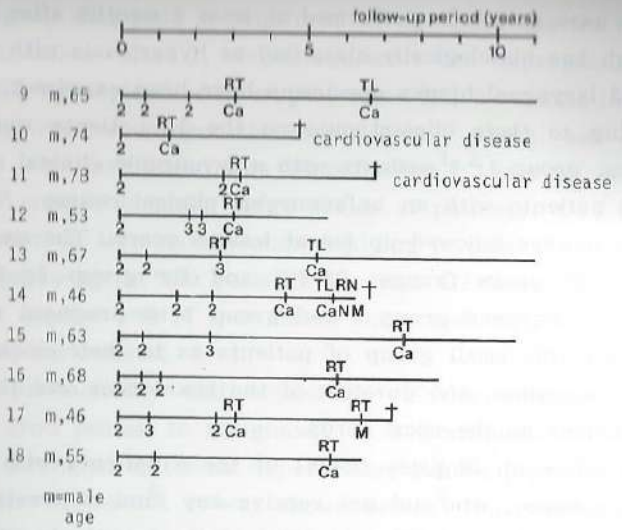

Fig. 2 Follow-up of 10 patients with an initial class II lesion, subsequently developing an invasive carcinoma (=Ca) RT=radiotherapy/TL=total laryngectomy/N=necknode metastasis/M=distant metastasis/RN=radical neck-dissection/2=classified in class II (=squamous cell hyperplasia with atypia)/3=class III (carcinoma in situ)/†=died.

(Fig. 2). Nine patients were then irradiated and in case of a recurrent carcinoma a total laryngectomy without (no. 9) or with a radical neck dissection (no. 14) was undertaken. In the last patient large metastases to the superior mediastinum were found during the operation. He died in a short while from these metastases. Two patients (nos. 10, 11) died 3 and 3½ years later, after the radiotherapy from cardiovascular diseases. Four of the 10 patients with unfavourable clinical follow-up (nos. 12, 13, 15, 17) on histological examination first progressed to carcinoma in situ. One patient (no. 13) received for this reason a full course of radiotherapy, but in spite of this, 2½ years later, his lesion was histologically proven to have progressed into an invasive laryngeal carcinoma. A total laryngectomy was then performed. In patient no. 17, who was first irradiated for an invasive carcinoma, nearly 3 years after the radiotherapy metastases were found in the superior mediastinum. He was treated with a full course of irradiation, but he died after 1 year from the metastases.

Histopathology

The 52 laryngeal biopsy specimens were fixed in 4% buffered formalin for 18-36 hours, embedded in paraplast, cut at 4-6 μm thick and stained with haematoxylin and eosin. All slides were blindly reviewed by two of us (JD, PO) and classified into the class (Kleinsasser's classification[1,2]) with squamous cell hyperplasia with atypia (n=47) or as carcinoma in situ (n=5). In class II there is a thickening of the epithelium, para- and dyskeratosis and a moderate number of mitoses is seen. The atypia is defined as individual cells with nuclear aberrations; the nuclei may be enlarged, irregularly shaped or may have abnormal staining. There might be a significant loss of polarity and in class II lesions the atypia does involve the full height of the epithelium. In case of a class III lesion - carcinoma in situ - there is a full thickness epithelial replacement by atypical cells.

Morphometry

Pathological epithelial changes often show focal variations, therefore representative areas (i.e. the most atypical areas) are selected for the morphometrical analysis. In each of the two cell layers (basal and superficial) of the epithelium, fifty nuclei, chosen at random, were measured.

In the basal cell layer nuclei following the basal membrane and in the superficial layer nuclei restricted to the superficial layers of 3-5 cells are chosen. Measurements were performed after projection of the slides with a projection microscope (at a total magnification of 1000 x) on a graphic tablet (Bit Pad One, Summagraphics Corp.). The selected morphometrical features were calculated from each measured case.

The following 5 parameters, which were selected in a previous study using the stepwise discriminant analysis[7], were calculated for their most discriminative power.

1. The mean nuclear contour index basal layer (NCI bas.l.); NCI: $\frac{\text{Perimeter}}{\sqrt{\text{area}}}$; its value increases when nuclear irregularity increases, the minimal value is found for a perfect circle: 3.545.
2. The mean nuclear area superficial layer (μm^2) (n. area sup. l.).

3. The standard deviation of the nuclear area basal layer (sd. n. area bas.l.).
4. Total number of nuclei per (arbitrary) unit area in the basal layer (nuclear crowding; n. crowd. bas.l.).
5. Nuclear crowding in the superficial layer (n.crowd. sup.l.).

The prognostic formula (F) was: 4.71 x NCI bas.l. + 0.01 x n. area sup.l. + 0.23 x sd. n. area bas.l. + 0.16 x n. crowd. bas.l. + 0.06 x n. crowd. sup.l. - 31.25; where $F \geq 0$ implies: prognostic unfavourable and $F < 0$ means: prognostic favourable.

The morphometrical measurements for the preceeding study[7] were performed on the initial biopsy specimens, histologically all classified as squamous cell hyperplasia with atypia. In the present study also the secondary and further biopsies will be analysed morphometrically, on the basis of the 5 selected features. This study includes 26 microscopical slides in group 1 and group 2 each. The biopsy specimens on which an invasive laryngeal carcinoma was diagnosed were excluded from this investigation.

RESULTS

In Table I and Table II the probability of classification by morphometry (using a linear discriminant analysis) in the prognostic favourable group and in the prognostic unfavourable group is shown.

In the non-progressive group, patients nos. 2, 3 and 6 (Table I) showed prognostically a rather favourable clinical course. Although patient no. 2 and 6 once, on the second biopsy, were classified morphometrically as prognostic unfavourable, the last biopsies were classified as prognostic favourable. Patient no. 6 died 6 months after the last biopsy from a bronchial carcinoma. Patient nos. 1, 5 and 7 were on their ultimately taken biopsies, classified as prognostic unfavourable; so morphometrically there was progression of the degree of atypia. Patient no. 5 died 9 months after the ultimately taken biopsy from a myocardial infarction. Two patients (nos. 4 and 8) were three times classified as prognostic unfavourable, but showed no progression to invasive carcinoma later on. For patient no. 4, the classification results by majority fall into the unfavourable group. It should be noted that 3 of the 4 biopsies show a classification probability of 54%, 57% and 56%, indicating

Patient no.	Morphometrical classification: Progn. Fav.	Progn. Unfav.	First biopsy: Progn. Fav.	Progn. Unfav.	Second biopsy: Progn. Fav.	Progn. Unfav.	Third biopsy: Progn. Fav.	Progn. Unfav.	Fourth biopsy: Progn. Fav.	Progn. Unfav.
1	80%		73%				78%			
2	74%				87%		93%			
3	97%		99%							
4				54%		57%	99%			
5	73%		87%				81%			
6	90%				82%		53%		73%	
7	67%				73%					
8			86%		90%			97%	73%	

Table I. A posteriori probability of classification by morphometry, using 5 nuclear parameters, in a prognostic favourable (=Progn.Fav.) or in a prognostic unfavourable (=Progn.Unfav.) group. Histologically the first and next biopsies of these 8 patients were classified as squamous cell hyperplasia and atypia of the laryngeal epithelium.

Patient no.	First Biopsy		Second biopsy		Third biopsy	
	Progn. Fav.	Progn. Unfav.	Progn. Fav.	Progn. Unfav.	Progn. Fav.	Progn. Unfav.
9		63%		100%		100%
10		97%				
11		96%	63%			
12		97%	carcinoma in situ: 100%		carcinoma in situ: 100%	
13	91%			70%	carcinoma in situ: 100%	
14		69%	96%		61%	
15		90%		90%	carcinoma in situ: 98%	
16	89%			98%	90%	
17		84%	carcinoma in situ: 100%			69%
18		89%	57%			

Table II. A Posteriori probability of classification by morphometry in a prognostic favourable (=Progn.Fav.) or in a prognostic unfavourable (=Progn.Unfav.) group, using 5 nuclear parameters. On the basis of the first biopsy the lesions of these patients were histologically classified as laryngeal squamous cell hyperplasia and atypia. The second and/or third biopsies were histologically classified as atypical hyperplasia or as carcinoma in situ.

considerable doubt in classifying. The remaining biopsy, the third one, on the contrary showed great certainty for classification in the prognostic favourable group. For patient no. 8 the first 3 lesions were classified as prognostic unfavourable with high probabilities. The last biopsy specimen was, however, classified in the prognostic favourable group. It could be so that the one but last biopsy examination had been "radical".

Six patients of the progressive group (nos. 9, 10, 12, 13, 15, 17; Table II) showed a clear morphometrical follow-up with almost all lesions classified in the prognostic unfavourable group, or as carcinoma in situ. The lesions histologically classified as carcinoma in situ were tested on the basis of an earlier assessed function[6] for discrimination between lesions histologically classified as squamous cell hyperplasia with atypia and as carcinoma in situ. In this study the lesions classified histologically as carcinoma in situ (patient no. 12, second and third biopsy; no. 13, third biopsy; no. 15, third biopsy; no. 17, second biopsy) were morphometrically all correctly classified.

In 4 patients of the progressive group (nos. 11, 14, 16, 18) the last biopsies, before an invasive carcinoma was demonstrated, were morphometrically classified in the prognostic favourable group. In patient no. 11 the last biopsy with hyperplasia and atypia was classified as prognostic favourable with a rather low probability (63%). This was also the case with the last hyperplastic biopsies of patients nos. 14 and 18 (61%, 57% respectively).

Even after the histopathological review of all follow-up lesions of patient no. 16, no satisfactory explanation can be given for the "misclassification" of the biopsy, taken before an invasive carcinoma developed.

DISCUSSION

Other morphometrical studies of the epithelia of the larger airways are concerned with changes in nuclear and cell parameters during development of metaplasia and dysplasia in bronchial and nasal epithelium.

Automatic detection of epithelial thickness, volume density of intracellular mucus and the number of nuclear profiles per unit area of the

sectioned epithelium provided objective separation of various types of bronchial epithelium (normal, mucous and metaplastic), as was found by Betram and Rogers[9].

Alterations of the nasal epithelium were studied by Boysen and Reith, who presented a morphometrical model for the evaluation of morphological alterations in the basal layer[10 11 12 13]. Among the several features evaluated the mean nuclear transverse diameter, the sum of the longitudinal and transverse nucleolar axes and the basal cell width (the cell's attachment to the basement membrane) emerged to be the best discriminators for grading metaplastic and dysplastic changes of the various types of precancerous epithelial transformation of the nose[13].

A similar problem as in the underlying report, was addressed in a study by Hellquist et al.[14]. In order to obtain an objective and reproducible classification and to reduce the subjectivity of the judgement, they examined different laryngeal epithelia (such as: hyperplasia and different types of dysplasia - mild, moderate, severe) with photometry. No differences were found in the nuclear DNA values for moderate dysplasia (more or less comparable to Kleinsasser's class II) with or without a later development of severe dysplasia or carcinoma in situ. They concluded that long term follow-up has to be performed to evaluate the biological significance of all these different lesions.

This study is part of a larger project where morphometry was applied for a more reliable assessment of the various types of nuclear atypia, as in hyperplasia with atypia and carcinoma in situ of the laryngeal epithelium. In the first study we described a function for discrimination between squamous cell hyperplasia with atypia and carcinoma in situ[6]. A set of nuclear features was thereafter selected by discriminant analysis to classify lesions with squamous cell hyperplasia as to their favourable or unfavourable prognosis[7], as Colgan has performed for marked atypical endometrial hyperplasia[15]. For differentiation of the two groups we found, the nuclear area in the superficial layer, the nuclear crowding in the basal and superficial layer, the nuclear irregularity in the basal layer (nuclear contour index) to be increased and the nuclear anisokaryose in the basal layer (as expressed in the standard deviation of the nuclear area), to be less pronounced in case of prognostic unfavourable lesions, with marked atypia, than in the prognostic favourable group of lesions with mild nuclear atypia[7]. In light microscopical examination, however, the differentiation proved to

be difficult.

In the present study the selected nuclear features were tested for their ability to predict the likelihood of progression from squamous cell hyperplasia with atypia to invasive carcinoma, in individual patients with longterm follow-up.

In evaluating the results we have assigned each biopsy either to the group of prognostic favourable or to the group of prognostic unfavourable. A margin of doubt, leaving a case unclassified when there is a reasonable likelihood on both groups (e.g. a classification probability between 40-60%) has not been used. Classification probabilities in the neighbourhood of 50% are to be considered questionable. When forcing the morphometrical results into either one group, the concordance with the a priori classification is in the non-progressive group 58% (15 out of 26) and in the progressive group 73% (19 out of 26).

The results of this classification as displayed in the Tables I and II are not always consistent with what one would expect on the basis of the clinical course. The classification sometimes appeared to be reversible. In some patients a prognostically unfavourable morphometrical classification is followed by a prognostically favourable morphometrical classification in the sequential biopsy. In these cases the "premalignancy" of the lesion may have been removed radically by the prior biopsy or the lesion removed is not representative. This is also possible in patients, were the ultimate morphometrical classification was unfavourable, but in whom the clinical outcome was favourable (e.g. nos. 1, 4; Table I). In some cases the biopsy examination cannot be regarded as representative for the unfavourable clinical outcome (sampling error), when - as in patient no. 11; Table II - within a few months after the morphometrical favourable examination an invasive laryngeal carcinoma has developed.

For a valuable morphometrical classification of lesions of individual patients, the results as presented in the underlying report still are insufficient. This is due to the mentioned intrinsical problems (sampling error, reversibility in the classification), the limited dataset (small samples of untreated patients) and the in general poorer classification results for teststudies.

A prospective study on greater samples of untreated patients with lesions histologically classified as class II and class III would have to be carried out in order to improve the morphometrical classification proba-

bilities for the individual patients. However, it will not be easy to realize this, because leaving class III patients untreated is justified only under the following strict conditions: the lesion is limited to one vocal cord and can macroscopically be removed in toto at initial microlaryngoscopy; patient and anatomy allow for indirect laryngoscopy to be carried out easily during follow-up, and patient should comply with regular follow-up visits and should stop smoking. The result of such a study may even be selection of a new set of discriminating features.

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Chapter VI

CLOSING REMARKS AND PERSPECTIVES.

From the foregoing chapters it is clear that classification of the so called precancerous lesions of the laryngeal mucosa is difficult, especially for class II and class III lesions. Reproducible classification is important because of the different management policies for class II and class III lesions. Moreover, indentification of patients in the class II group with an unfavourable clinical course is desirable.

In this study morphometry was applied in order to determine whether morphometrical analysis of some epithelial characteristics can contribute to a reproducible histopathological classification of squamous cell hyperplasia of the larynx. Microscopical slides of patients with hyperplastic laryngeal lesions were projected on a graphic digitising tablet. Measurements were made of the nuclei (area, contour index, polarity, crowding) in the three layers of the epithelium and of the epithelial width and proved to be reproducible. The average time to perform the measurements in one slide is 15-20 minutes. The data were analysed statistically and a function for discrimination between squamous cell hyperplasia with atypia (class II) and carcinoma in situ (class III) was developed (chapter III).

As in other retrospective clinico-pathological analyses, many patients in this study had previously been treated, especially those with class III lesions. This hinders long term follow-up and makes comparison with untreated patients assigned to class II difficult. Such previous treatment of patients classified as class III is not really a problem, since there is more or less agreement amongst clinicians that class III lesions in general should be treated. In histopathological practice differentiation between class II and class III lesions was not easy. Identi-

fication of patients with class II lesions who subsequently developed invasive carcinoma (about 20-25% of the untreated class II patients) proved to be difficult on histopathological grounds.

This study has shown (chapter II) that the majority of the patients with a class II lesion, who later developed an invasive carcinoma, did not go through a morphologically identifiable carcinoma in situ stage in the biopsies taken. Further, we were able to select in a retrospective study from a learning set of class II patients, those morphometrical parameters, which identified class II patients, who later developed invasive carcinoma. However, when these selected parameters formulated in a numerical decision rule, were tested on another set of class II lesions the results proved to be insufficient. It should be noted that this study was hampered by several factors: the "premalignancy" of the lesion may have been removed radically by the prior biopsy, or the lesion removed is not representative (sampling error). Retracing these factors is difficult because the localization of the lesions had not been recorded precisely. On the basis of these results it is concluded that in future it is mandatory to describe these hyperplastic lesions accurately and to record their localization photographically, now that the technical equipment is available.

A prospective study on a larger number of biopsies may improve the value of the morphometrical classification in identifying the patients of class II with an unfavourable prognosis. Longterm (life long) follow-up of the patients is necessary, because even after 10 years squamous cell carcinoma has developed in patients with squamous cell hyperplasia with atypia (class II).

PERSPECTIVES

Besides morphometry the otolaryngologist and the pathologist still need other objective methods to predict the clinical behaviour of class II lesions. In this paragraph possible techniques will be discussed shortly, which may be used to solve the question which patients of class II have an unfavourable clinical course.

Lectin labelling of exfoliating laryngeal mucosal cells. Davina et al.[1] have shown that the labelling percentage of exfoliating cells of the uterine cervix with the lectin concanavalin A labelling method, decreased gradually with increasing atypia of the epithelium. When the course of the labelling percentage of a patient is followed longitudinally, the method may contribute to the prediction of the future behaviour of a cervical intraepithelial neoplastic lesion. If the same results are obtained, when this method is applied to exfoliating flattened cells of the laryngeal epithelium, it may help to more accurately classify hyperplastic lesions of the larynx.

In this respect evaluation of the presence of epidermal growth factor receptor in cells of the laryngeal mucosa as demonstrated by monoclonal antibodies may also be of importance[2]. It has been shown for carcinomas that the expression of these receptors is increased[3]. Perhaps a quantitative analysis of the epidermal growth factor receptors on the cells of class II lesions might yield prospective information.

Recently Tan et al.[4] have described a low molecular weight factor related to retroviral P15E in sera of patients with laryngeal carcinoma. This factor influences negatively monocyte chemotactic responsiveness and thus may have a negative effect on tumor rejection by the immune system. Whether the presence of this factor is of significance in identifying patients with class II lesions, who have an unfavourable prognosis, remains to be evaluated.

More studies now seem to indicate that viruses play an important role in neoplasia[5]. Recently such a role has been claimed for human papilloma virus type (congress on HPV, Helsinki, Finland, 1985) in the genesis of squamous cell carcinoma of the larynx. When these results can be confirmed by others it might well be that like in squamous cell lesions of the cervix[6 7], the presence of certain types of HPV viruses in the laryngeal mucosa identifies patients at risk for laryngeal carcinoma. In this way in situ hybridization for the detection of HPV viruses in laryngeal mucosa may prove to be useful in the patients.

With DNA cytometry; aneuploidy is a well recognized feature of human tumours. Quantitative DNA analysis reflects to the total chromosomal content of tumour cells and can now be determined rapidly and reliable using flow cytometry. In a study of cervical intraepithelial neoplasms[8] it was shown that cells exhibiting mild to moderate dysplasia were invariably diploid while areas of severe dysplasia and carcinoma in situ were usually aneuploid, with a frequency approaching that of invasive cervical carcinoma. In a study on dysplastic squamous epithelial lesions of the larynx[9], using static DNA cytophotometry and compu-

ting the results according to an algorithm for DNA diagnosis, within the group of histologically mild to moderate dysplasias the patients could be identified, who were proven to have a carcinoma in the follow-up.

Further studies, making use of the mentioned techniques, will contribute to identification of patients with class II lesions, who are at risk for invasive laryngeal carcinoma.

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SUMMARY

This thesis attempts to elucidate some of the encountered problems in clinical practice, regarding the classification of lesions with squamous cell hyperplasia of the laryngeal epithelium. In literature is little agreement among authors for the definition of these kinds of laryngeal lesions. In this thesis a three grade classification, introduced by Kleinsasser with modifications of Delemarre, is used. According to the authors hyperplastic changes in the epithelium can be classified as to the degree of atypia: class I, simple squamous cell hyperplasia; class II, squamous cell hyperplasia with atypia; class III, carcinoma in situ. Retrospective clinical analysis have shown that Kleinsasser's classification has prognostic significance: future development of carcinoma correlates with increasing class I to III.

However, in practice, problems in the use of this histopathological classification are converging in the transition of class II to class III. In this study morphometry was applied to determine the features of atypia, in order to improve the histopathological classification of these lesions.

In chapter I literature on hyperplastic squamous cell lesions of the laryngeal mucosa is reviewed. The value of Kleinsasser's classification system is discussed, emphasizing the differential diagnostic problems.

In chapter II a retrospective clinico-histopathological analysis is presented, comprising 200 patients seen between 1963-1981 in the Netherlands Cancer Institute and the Free University Hospital and followed for a mean period of 8.4 years (range: 5-21). The most remarkable finding at revision of the microscopical slides was, that about one third of the cases, who at revision were assigned to class II, originally were classified as carcinoma in situ, while the reverse hap-

pened for just a few cases. The problem thus was overclassification, which probably resulted in overtreatment in individual cases. The reproducibility of the classification, however, may be improved when strict histopathological criteria for class II and class III are used. For untreated cases (especially as to class II) the prognostic significance of Kleinsasser's classification, which was shown in earlier studies was confirmed.

Morphometry was applied on microscopical slides from patients with squamous cell hyperplasia of the laryngeal epithelium (chapter III). Using morphometrically assessed features of the nuclei and the epithelial width, one can easily distinguish between class I and class II and class I and class III. Differentiation between class II and class III proved to be more difficult. The findings suggest that within class II two groups with different prognoses could be distinguished by morphometry. Unfortunately, the numbers of patients within these two groups were too small to allow for definite conclusions.

Therefore microscopical slides of only non-treated patients (i.e. no more than endoscopic biopsy) were selected (chapter IV). Most of the patients were classified as class I and class II, only a few as class III. Patients with a class II lesion were divided into 2 groups on the basis of a favourable or unfavourable clinical course. With linear discriminant analysis applied on the morphometrical features, a clear distinction between the 2 aforementioned groups of class II patients was found. Using 5 selected nuclear features, 79% of the cases of the 2 groups could be correctly classified with an average classification probability of 0.82.

The selected nuclear features were tested (chapter V) for their ability to predict the likelihood of eventual progression from squamous cell hyperplasia with atypia to invasive carcinoma in individual patients with long term follow-up. This study has shown that morphometry is insufficient for a valuable morphometrical classification of hyperplastic laryngeal lesions of individual patients.

In chapter VI the perspectives for the clinician and pathologist are shown to achieve more accurate diagnostics and thereby more accurate treatment of these lesions.

SAMENVATTING

Dit proefschrift tracht enkele problemen op te helderen, die zich in de praktijk voordoen bij de classificatie van laesies van het larynxepitheel met plaveiselcellige hyperplasie. Uit de literatuur blijkt dat men het nauwelijks eens is omtrent de definitie van dit soort laesies. In dit proefschrift wordt de classificatie volgens Kleinsasser gebruikt, met wijzigingen daarin aangebracht door Delemarre. Volgens deze auteurs kunnen hyperplastische veranderingen in het epitheel op grond van de mate van atypie in drie klassen worden ingedeeld: klasse I, eenvoudige plaveiselcellige hyperplasie; klasse II, plaveiselcellige hyperplasie met atypie; klasse III, carcinoma in situ. Retrospectieve klinische studies hebben aangetoond dat de classificatie volgens Kleinsasser prognostische waarde heeft: de kans op het later ontstaan van een invasief carcinoom neemt toe van klasse I naar klasse III.

De problemen bij het gebruik van deze histopathologische classificatie blijken zich met name voor te doen in het grensgebied van klasse II en klasse III. In dit onderzoek wordt morphometrie toegepast om de kenmerken van atypie vast te stellen, met het doel de classificatie van deze laesies te verbeteren.

In hoofdstuk I wordt een overzicht gegeven van de literatuur over hyperplastische plaveiselcellige laesies van het larynxepitheel. De waarde van de classificatie van Kleinsasser wordt besproken, waarbij de nadruk wordt gelegd op de differentiaal-diagnostische problemen.

In hoofdstuk II wordt een retrospectief klinisch-histologisch onderzoek beschreven, dat 200 patienten betreft, die tussen 1963 en 1981 in het Antoni van Leeuwenhoekhuis en het Academisch Ziekenhuis van de

Vrije Universiteit te Amsterdam zijn onderzocht. Deze patienten werden gedurende een periode van gemiddeld 8.4 jaar (variërend van 5 tot 21 jaar) gecontroleerd. Bij herbeoordeling van de microscopische preparaten blijkt het meest opvallend dat ongeveer 1/3 van de laesies, die bij revisie zijn ingedeeld in klasse II, oorspronkelijk als carcinoma in situ waren geclassificeerd. Daarentegen was slechts een beperkt aantal laesies oorspronkelijk te laag ingedeeld. Een dergelijke te hoge classificatie, heeft in individuele gevallen waarschijnlijk tot overbehandeling geleid. De reproduceerbaarheid van de classificatie zou kunnen worden verbeterd, indien voor klasse II en klasse III strikte histopathologische criteria worden gehanteerd. Dit onderzoek bevestigt voor onbehandelde patienten (met name die met een klasse II-laesie) de voorspellende waarde van de classificatie van Kleinsasser.

Morphometrie is toegepast op microscopische preparaten van patienten met plaveiselcellige hyperplasie van het larynxepitheel (hoofdstuk III). Door gebruik te maken van morphometrisch vastgestelde kenmerken van de kernen en van de breedte van het epitheel, kan duidelijk onderscheid worden gemaakt tussen zowel klasse I en klasse II als klasse I en klasse III. De verschillen tussen klasse II en klasse III blijken ook met behulp van morphometrie moeilijk aan te geven. Wel kunnen binnen klasse II twee groepen met verschillende prognoses worden onderscheiden. Het aantal patienten van deze twee groepen is helaas te klein om aan dit deel van het onderzoek conclusies te kunnen verbinden.

Voor een vervolgonderzoek (hoofdstuk IV) zijn alleen microscopische preparaten uitgekozen van onbehandelde patienten; bij deze patienten waren slechts biopsieën genomen tijdens endoscopie. Het merendeel van deze patienten is ingedeeld in klasse I en klasse II, slechts enkele in klasse III. De patienten met een klasse II-laesie zijn in twee groepen onderverdeeld op grond van een gunstig of ongunstig klinisch beloop. Met een lineaire discriminantanalyse, toegepast op de morphometrisch bepaalde kenmerken, is een duidelijk onderscheid aan te geven tussen de twee eerder genoemde groepen van patienten uit klasse II. Van die twee groepen kan 79% juist worden geclassificeerd met een waarschijnlijkheid van gemiddeld 0.82, bij toepassing van vijf geselecteerde kern-kenmerken.

Deze kenmerken zijn getest (hoofdstuk V) op laesies met atypische plaveiselcellige hyperplasie van individuele patienten, die langdurig gecontroleerd zijn. Uit dit onderzoek blijkt dat morphometrie onvoldoende

zekerheid oplevert voor het juist classificeren van klasse II-laesies. De waarde van deze kern-kenmerken is te gering om in individuele gevallen een eventuele ontwikkeling van een invasief carcinoom te kunnen voorspellen.

In hoofdstuk VI worden perspectieven geboden aan de clinicus en de patholoog-anatoom om tot een nauwkeuriger diagnostiek en daardoor tot een zorgvuldiger behandeling van deze laesies te komen.


ZEGGU, JONGEMAN, WAT WIL JE WORDEN ALS JE LATER GROOT BENT?
DOKTER EN JURIST
WAAROM ALLETWEE?
VOOR HET GEVAL IK EEN SLECHTE DOKTER ZOU ZIJN!

VRIJE UNIVERSITEIT TE AMSTERDAM

MORPHOMETRY AND SQUAMOUS CELL HYPERPLASIA OF THE LARYNX

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de geneeskunde
aan de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus, dr. P.J.D. Drenth,
hoogleraar in de faculteit der sociale wetenschappen,
in het openbaar te verdedigen
op vrijdag 14 maart 1986 te 15.30 uur
in het hoofdgebouw der universiteit,
De Boelelaan 1105

door

PETER HENDRIK MARIE THERESIA OLDE KALTER

geboren te Bilthoven

drukwerkverzorging: OPTIMAX

Promotores	:	prof. dr. G.B. Snow
	:	prof. dr. C.J.L.M. Meijer
Copromotor	:	dr. J.F.M. Delemarre
Referent	:	dr. ir. A.W.M. Smeulders

STELLINGEN

behorende bij proefschrift:

"Morphometry and squamous cell hyperplasia of the larynx".

1. Met behulp van morphometrie kan de indeling volgens Kleinsasser van plaveiselcellige hyperplasie van het larynxepitheel worden verfijnd: binnen klasse II kan een groep met gunstig beloop worden onderscheiden van een groep met ongunstige prognose.

2. De reproduceerbaarheid van de classificatie volgens Kleinsasser kan worden verbeterd door de criteria voor de verschillende klassen strikt te hanteren. Dit zal leiden tot een geringer aantal te hoog geclassificeerde laesies, hetgeen klinische consequenties heeft.

3. Om beter inzicht te krijgen in het klinisch beloop van hyperplastische afwijkingen van het larynxslijmvlies, verdient het aanbeveling deze zowel bij de initiële diagnostiek als bij vervolg onderzoekingen fotografisch vast te leggen.

4. Elke patient bij wie ooit een plaveiselcellige hyperplasie van de larynx werd vastgesteld, dient levenslang te worden gecontroleerd wegens de verhoogde kans op het later ontstaan van plaveiselcelcarcinoom. Patienten met een klasse II en III laesie moeten bovendien worden gecontroleerd wegens de verhoogde kans op het later ontstaan van longcarcinoom.

5. Een overgevoeligheidsreactie type III speelt mogelijk een rol bij otitis media met effusie. In het middenoorslijmvlies zijn tot nu toe echter nog geen immuuncomplexen aangetoond.

6. Röntgenologische afwijkingen in een kaakholte van een kind vormen op zich geen indicatie voor een kaakspoeling.

7. Medische gegevens van consultatiebureaus voor jeugdgezondheidszorg mogen pas na inzage door en na toestemming van de ouders worden verstrekt aan andere instanties.

8. Het is niet raadzaam in de Wet op de Lijkbezorging een termijn op te nemen die de grens aangeeft waarna een te vroeg geboren kind levensvatbaar kan worden geacht. De wet moet slechts aangeven na welke termijn een bezorgingsplicht bestaat.

9. Het werkwoord "doorverwijzen" is een contaminatie die het taalgebruik van medici ontsiert.

10. Doordat de centrale overheid personeelsadvertenties voor hogere ambtelijke beleidsfuncties slechts in een beperkt aantal dagbladen laat opnemen, kan op lange termijn het gevaar ontstaan van een politiek onevenredige samenstelling van het ambtenarencorps.

11. Applaus bij concerten en toneelstukken devalueert als het te vaak overgaat in een staande ovatie.

P.H.M.T. Olde Kalter 14 maart 1986.