Symposium 10

Rev Esp Patol 1999; Vol. 32, Nº3: 325-333

Progress in laryngeal pathology

Organized by the ESP Working Group on Head and Neck Pathology
Chairperson: L. Michaels, *UK.* **Ca-chairpersons:** H.B. Heliquist, Norway and N. Gale, *Slovenia.*

Laryngeal precancerous lesions: Current diagnostic and prognostic considerations

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The diagnosis, treatment and prognosis of laryngeal epithelial changes, which are referred to here as epithelial hyperplastic laryngeal lesions (EHLL), depend almost entirely on their histological abnormalities. EHLL cover the whole spectrum of histomorphological changes with more or less expressed cellular and structural abnormalities with a preserved basement membrane (1). However, the lack of uniformity and inconsistency of the terminology, and the fact that the histological features and the biological behavior of these lesions are not always in accordance, have frustrated attempts to gain an internationally accepted classification of EHLL. It is not surprising that more than 20 classifications of EHLL can be found in the literature within the last three decades, however none has entirely fulfilled our expectations for daily practice in predicting evolution, particularly of high risk lesions (2-5). The majority of EHLL classifications have followed similar criteria to those in common use tor epithelial lesions of the cervix of the uterus. The three-graded system of cervical lesions, now most frequently called cervical intraepithelial neoplasia (CIN) I,II,III, has been applied to EHLL, with the possible addition of a fourth grade as carcinoma *in situ (6).* For EHLL, different terminologies have been created, such as keratosis with mild, moderate and severe dysplasia (7), mild, moderate and severe dysplasia 2, and laryngeal intraepithelial neoplasia with three grades (LIN I,II,III) (8).

Nevertheless, the etiopathogenesis of laryngeal cancer is more likely related to different noxious influences than those of the cervix of the uterus, in which mounting evidence links human papilloma viruses (HPV) types 16 and 18 with precancerous and cancerous lesions. In laryngeal carcinogenesis, not HPV infection, but rather cigarette smoking, alcohol abuse, hormonal disturbances, and various hazardous occupational irritants most probably trigger not yet entirely recognized pathways of genetic events (2, 5, 9-12). In addition to a different etiology, there are also distinct clinical and histological laryngeal specificities which require a specially devised EHLL grading system and not uncritically applied CIN classification. In laryngeal lesions, there is a pressing need to identity, in one biopsy, conditions with a substantial hazard of becoming malignant. Furthermore, no operation has been invented on the laryngeal mucosa corresponding to cervical cone excision, which removes the whole affected area and thereby reduces the need for accurately distinguishing between potentially and actually malignant intraepithelial cervical lesions. This separation is, in contrast, crucial in the larynx, where the two lesions are treated differently. Another requirement for EHLL classification is dividing the lesions into benign and potentially malignant. Such a separation is of essential importance in the clinical handling of patients and is not furnished by the dysplasia grade systems (5).

A classification of EHLL, proposed and tested in Ljubljana, Slovenia, for more than 25 years, does not follow the three-grade criteria used for classifying cervical lesions, but was devised to cater to specific clinical and histological laryngeal problems (3, 13). The Working Group on EHLL of the European Society of Pathology reevaluated and further formulated the histological criteria of the Ljubljana classification in November 1997 in London, UK, The system is divided into four grades as follows: simple (SH) and abnormal (AbH) hyperplasia form a benign group, atypical hyperplasia (AtH) is potentially malignant, and carcinoma *in situ* (CIS) a malignant lesion. SH is characterized by an augmented spinous cell layer while the basal layer remains unchanged. In AbH, basal and parabasal cells are augmented to the midportion of the epithelial thickness with a perpendicular orientation to the basement membrane. AtH, or risky epithelium, is characterized by a still preserved stratification of epithelial cells, which are enlarged and augmented with basal, and on rare occasions spinous type, differentiation. The nuclear/cytoplasmic ratio is increased and the nucleoli may be augmented in number. Mitoses are increased but not numerous, and are found within the two lower thirds of the epithelium; they are rarely, if ever, abnormal. Dyskeratotic cells are frequent, and apoptotic cells may be present. In CIS, which shows the features of malignancy without invasion, three distinct morphological changes are diagnostically important: loss of epithelial stratification as a whole, marked cellular alterations, and many mitotic feature, more than five per high power field (5).

The results of malignant transformation of individual grades of EHLL justify our proposal for dividing the lesions into benign and potentially malignant, according to their histological features. Simple and abnormal hyperplasias are considered benign forms with 0.7% (3/380 patients within 15 years) and 1% (4/414 patients within the same period) of malignant transformation, respectively. AtH is a precancerous lesion in the essential meaning of the word, with 9.5% (101105 patients) of malignant alteration within 15 years (3). According to other studies of EHLL, patients with AtH or severe dysplasia present the most threatening group associated with the highest risk of cancer development ranging from 19-28% (7,14).

Laryngeal precancerous lesions have no specific macroscopic appearance, and are variously referred to as chronic laryngitis, leukopiakia, keratosis, erythroplakia, dysplasia, etc. The surface morphology and keratin layer formation of these lesions has no specific meaning, nor any significant relationship with their malignant potential (3, 4). Histopathological diagnosis informs a clinician well of how to treat patients with benign, potentially or actually malignant lesions. On the other hand, reliable evidence is still lack-

ing on how to separate high-risk from low-risk precancerous lesions. Such information would assist in predicting which AtH will regress, remain stable, or progress to invasive cancer, and would thus influence the mode of treatment.

The studies presented here, based on the light microscopic level, may be considered to be clinically feasible methods for daily routine practice in determining the risk level for laryngeal cancer development.

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Clonality studies in multiple head and neck cancers: p53 mutations compared with LOH at 3p, 9p and 17p loci

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Introduction

Patients wjth head and neck squamous cell carcinoma (HNSGC) are at risk of developing additional tumors in the head and neck (1). However, HNSCC patients also frequently develop local reCurrences or locoregional metastases (2). Differentiation between metastasis or recurrence of the primary tumor *versus* second tumor may be difficult as all lesions have the histological appearance of squamous cell carcinoma. Differentiation between these possibilities, however, carries important differences in therapeutic and prognostic consequences. Therefore, diagnostic modalities other than histopathological ones are needed to distinguish between local recurrence and metastasis on the one hand and second tumor on the other. Molecular biological detection techniques may be useful in these cases. Several genetic markers have been used for assessing the clonal relationship between separate HNSCCs occurring in individual patients. Among these are loss of heterozygosity (LOH) patterns at loci 3p and 9p. These changes have been shown to occur early in carcinogenesis (3, 4), but as it has been demonstrated that they may differ among primary tumors and their matched lymph node metastases, it is obvious that they do not meet the criterion of stability during tumor progression and metastasis (5, 6). Furthermore, p53 mutations have been employed as a clonal marker. This seems to be promising, p53 being mutated in a high percentage of HNSCCs and showing a huge variability in its mutations (7). However, p53 will only be useful as a clonal marker in HNSCC when mutations develop before metastasis has occurred and they are not lost during tumor progression. This can be demonstrated by investigating whether a specific p53 mutation is consequently found in a primary tumor and its matched lymph node metastasis. Literature on this issue yields conflicting data. In some studies, complete concordance was observed (8-17), but different p53 mutations in primary tumor and lymph node metastasis have also been reported (11-13, 18, 19). Tumors may contain different clones, with different expression of metastatic potential. Discordancies in p53 mutations between primary tumor and lymph node metastasis mean that studies in which different p53 mutations in different neoplastic lesions are supposed to indicate an independent origin of those lesions (8, 10, 11, 15, 17, 20, 21) are at least premature: the possibility that a tumor has changed its p53 mutation status during progression by acquiring new mutations or losing initially present mutations cannot be excluded.

Materials and methods

We recently developed a p53 mutation analysis strategy consisting of direct sequencing full-length p53 mRNA as well as DNA from the mutated exon followed by screening for mutations already identified by DNA sequencing. This strategy proved to be very sensitive and resulted in a p53 mutation percentage of almost 100% in an unselected sample of HNSCCs (22). In the present study, we applied this technique to study 15 primary HNSCCs with matched lymph node metastasis to determine whether p53 mutations are stabile during metastasis. We also compared loss of heterozygosity at loci 3p, 9p and 17p in this same series to confirm or refute differences in LOH patterns at these loci emanating during metastasis and tumor progression. Furthermore, tumor tissue from eight cases with multiple primary HNSCCs, from four autopsied HNSCC cases with disseminated disease and from three cases with surgically treated HNSCC as well as lung SCC were subjected to the same p53 mutation analysis to obtain a full impression of the usefulness of p53 mutations when investigating the clonal relationship between several HNSCC manifestations in an individual case. Studies concerning 3p and 9p LOH for the three latter groups are still in progress and will be presented at the symposium.

Results and discussion

In all cases of primary tumor and matched lymph node metastasis, p53 mutations were identical. These results are in agreement with the studies referred to in the Introduction section, in which the same concordance between primary tumor and lymph node metastasis was observed. It appears that in HNSCC carcinogenesis, p53 mutations occur before a lesion metastasizes and are maintained during metastasis. Although in the process of tumor progression, additional mutations may occur or mutations may be lost, these phenomena apparently do not influence the p53 status. Similar findings on consistency in p53 status in the autopsied cases confirm this stability of p53 status during tumor progression and metastasis, In the cases with multiple HNSCCs, all lesions had different mutations. These data support the assumption that different p53 mutations in different HNSCCs are due to an independent origin of these tumors. The alternative monoclonal theory (23) appears to be less likely. This theory states that new tumors could be the result of micrometastatic foci and that the difference in p53 mutations in different lesions can be explained by assuming that p53 mutations are either lost or acquired during metastasis. In this case, similar changes would occur during the development of lymph node metastasis and result in differences between p53 mutations in primary tumor and lymph node which disagrees with our observations. Moreover, p53 mutation analysis to assess cional relationship appears to be superior to LOH studies. In the 15 cases of primary tumor with matched lymph node metastasis, no differences in LOK were observed which suggests that these markers are stabile during tumor progression; however, in this rather limited sample, the same LOH pattern was observed in HNSCCs from different patients. Therefore, the occurrence of similar LOH patterns in anatomically distinct tumors in an individual case may be due to chance and cannot be considered proof for a common clonal origin. The three cases with clinically separate primary SOCs in the head and neck region and the lung serve to illustrate the application of p53 mutation analysis in clinical pathology. Different p53 mutations were observed in one case and identical p53 mutations in the two others, thus suggesting lung metastasis from HNSCC in the two former cases and a second primary tumor in the latter case.

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Laryngeal spindle cell, verrucous and basaloid squamous carcinoma

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Spindle cell carcinoma

This variant of squamous cell carcinoma (SCC) is histologically characterized by a SCC and another underlying or adjacent spindle cell or pleomorphic component (1). Most but not all spindle cell carcinomas grow rapidly, are polypoid and bulky. They constitute approximately 1% of all malignant laryngeal neoplasms, In the upper respiratory tract, the larynx is the most common site, and

within the larynx, the true vocal cords (2). Radiotherapy will not induce an anaplastic carcinoma, Tumors with histology compatible with spindle cell carcinoma are not homogenous but comprise the following: 1) spindle cell carcinoma (SOC associated with malignant spindle cells that are demonstrably epithelial); 2) squamous cell carcinoma with pseudosarcomatous stroma in which a SCC is associated with atypical but non-neoplastic fibroblastic or fibrohistiocytic proliferation (Lane's pseudosarcoma); and 3) true examples of carcinosarcoma as in the three listed below.

i) Spindle cell carcinoma may histologically show a wide spectrum ranging from pleomorphic sarcoma suggestive of rhabdomyosarcoma, or dense fibrosarcoma-like structures, to more loose vascular structures. The spindle-shaped cells may vary in appearance, sometimes being very pleomorphic and bizarre. The spindle cells are of squamous origin, may be closely packed, and may sometimes even have a clear cell appearance. Abundant collagen is a frequent finding, whereas osteoid or cartilaginous tissue are not (2, 3). Mitoses are infrequent. Bizarre giant tumor cells are common while multinucleated giant cells are rare. The surface epithelium is often ulcerated and dysplastic. In many cases, but not all, one can see a diffuse connection with the overlying epithelium. In yet other cases, the lesion is associated with a readily recognized invasive SCC of the overlying epithelium. The blood vessels show no tendency towards a radial arrangement. In most cases the spindle cells stain positively for cytokeratins, and may also have a dual positivity for vimentin (2, 4). The spindle cells are often nondiploid, and have a lower mean per cent MIB-1 staining than the squamous component (4).

ii) The second type is a SCC with pseudosarcomatous stroma (Lane's pseudosarcoma) (5). The spindle cells are atypical and bizarre, but are non-neoplastic. The spindle cells are of fibroblastic or fibrohistiocytic origin, and negative for cytokeratins. There are, however, rare occasions when no SCC is to be found but only *in situ* carcinoma.

ii) The third type, carcinosarcoma, is very rare in the larynx. Laryngeal carcinosarcoma is as carcinosarcoma elsewhere in the body, *i.e.,* a sarcoma coexisting with squamous cell carcinoma.

Verrucous squamous cell carcinoma

Verrucous squamous cell carcinoma (VSCC) is an extremely well differentiated variant of squamous cell carcinoma (1, 6), which may be erroneously diagnosed as it lacks several of the conventional features of epithelial malignant neoplasia. It represents 1-2% of laryngeal carcinomas (7). It is usually superficial, bulky and presents as a warty papillomatous surface. Most tumors are found in the glottis (8). VSCC shows slow growth and eventually local invasion but rarely, if ever, metastases. The low-grade malignancy of this neoplasm allows for conservative therapy and neck dissection is not indicated. Radiotherapy gives good results, and does not induce anaplastic carcinoma (9). Microscopically the tumor consists of a well-differentiated epithelium with keratinized surface, and apparent absence of both dysplasia and stromal invasion. The rete ridges are blunt ended and irregular. Proliferating squamous masses often bulge outwards in a papillary fashion. Superficially abundant keratin is present, often displaying an externally pointed appearance, *i.e.,* the "church spire" effect. Typically, there is a dense, mononuclear cell exudate beneath the tumor processes. Under high power, the basal cells are crowded and composed of prominent cells, often with vesicular nuclei and showing a single eosinophilic nucleolus (9). The cells of the intermediate zone of VSCC appear to be larger than those of the same zone in squamous papilloma (10). Image analysis has demonstrated a significant difference between the sizes of the intermediate cells in these two conditions; a mean cell area greater than 300 μm^2 squared supports a diagnosis of VSCC whereas an area less than 250 μm^2 supports a diagnosis of squamous papilloma (11). Examination of VSCCs and papillomas for the presence of human papilloma virus (HPV), using *in situ* hybridization with DNA probes, has revealed that papillomas may be related to HPV, but VSCCs are likely not (42 of 42 papillomas were positive for HPV 6/11, while none of 11 VSCC contained demonstrable HPV) (12). Verrucous SCC of the larynx should thus be distinguished from squamous papilloma. Discrimination can also be made from papillary squamous cell carcinoma, which is an invasive SCC with an exophytic papillary component (1). This latter distinction is less important because papillary SCC has a clinical behavior similar to verrucous SCC.

Basaloid squamous cell carcinoma

This is a high-grade SCC which is a bimorphic carcinoma consisting of a mixture of basaloid and squamous cell components (1). Basaloid SCC was recognized as a separate entity in the upper respiratory tract only a decade ago (13). Various terms have been used to designate this neoplasm, and when the tumor occurs in the anal canal, it has been called cloacogenic carcinoma, In the upper respiratory tract it is predominantly found in the hypopharynx and base of tongue. Basaloid SCC is a tumor of high-grade malignancy, and should be distinguished from i) adenoid squamous cell carcinoma (low-grade), and ii) adenosquamous cell carcinoma (high-grade), Adenoid squamous cell carcinoma shows pseudoglandular lumina or spaces resulting from acantholysis of the tumor cells. There is no true glandular differentiation and thus no mucin production. Adenosquamous cell carcinoma has histological features of both adenocarcinoma and SCC. Laryngeal basaloid SCC often appears as an exophytic, polypoid and centrally ulcerated mass. Microscopically, the basaloid component is usually the more prominent, and consists of small cells with hyperchromatic nuclei and scanty cytoplasm. The cells are arranged in lobular masses or cords, often with small cystic spaces containing mucoid material, which stains with periodic acid-Schiff stain and/or Alcian blue. There is peripheral palisading, focal squamous differentiation, often numerous mitoses, and areas of necrosis. Stromal hyalinization is a common finding, The squamous component, which may be invasive or in *situ,* can be defined by two or more of the following: i) keratin pearl formation; ii) individual cell keratinization; ii) intracellular bridging; and iv) cells arranged in a pavement or mosaic pattern (13, 14). Basaloid SCC is positive for cytokeratins, often for EMA and CEA, and may also show positivity for, *e.g.,* NSE and S-100 (15, 16). The differential diagnosis is primarily with adenoid cystic carcinoma, but also with neuroendocrine carcinoma (small cell). High molecular weight cytokeratin reactivity is reported to more consistently differentiate basaloid SCC from small cell undifferentiated carcinoma than reactivity with neuroendocrine markers (17).

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Adenocarcinomas and salivary gland neoplasms of the larynx

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Introduction

Minor salivary gland tumors of the larynx are rare; only a few large series have been reported from a single institution. Therefore, details of their clinical and pathological behavior come only through composite analysis of small series. Less than 1% of the epithelial malignancies of the larynx are of salivary gland origin.

Subepithelial and intraepithelial glands

The sites of origin of the salivary gland neoplasms of the larynx follow the anatomical distribution of the larynx subepithelial glands and the intraepithelial mucous glands. Approximately two-thirds of the adenoid cystic carcinomas are in the subglottis, The other carcinomas, in contrast, are rarely subglottic, with supraglottic and transglottic involvement being nearly equal. The lower part of the glottic region shows the greatest differences in density of submucosal glands: 13 glands/cm2 on the vocal cords to 128 glands/cm2 on the false vocal cords and medial wall of Morgagni's sinus. The greatest concentration of glands is in the saccule (139 glands/cm^2). There is a Very low density of glands in the extrinsic laryngeal regions. A typical intraepithelial gland is made up of 15-30 mucus-secreting cells with a structure like that of goblet cells. They extend from the epithelial surface down toward the basement membrane, on which they may rest, but do not penetrate. Irregularly distributed in the larynx, intraepithelial glands are most numerous in the supraglottis and least numerous in the subglottis.

Salivary gland neoplasms

With the exception of adenoid cystic carcinomas, salivary-type carcinomas are rare in the larynx. Even pleomorphic adenomas are almost curiosities in this organ.

Benign neoplasms

Benign laryngeal neoplasms are extremely unusual with exception of oncocytic lesions.

Oncocytic lesions (metaplasia, hyperplasia, oncocytoma

Oncocytic lesions of the larynx occur most often in patients aged 50-80 years. A slight predominance of male patients exists. These lesions are most often located in the false Vocal cord or laryngeal ventricle areas. The microscopic findings in most cases suggest oncocytic metaplasia and cystic hyperplasia rather than neoplasia. Well-defined, columnar, oncocytic epithelium arises from seromucous ducts or acini and subsequently expands into cystic structures. Papillary growths into the cystic spaces are common. The process seems to be multifocal in the large majority of cases, and this contributes to the impression that this is a metaplastic process.

Pleomorphic adenoma

Certainly, the diagnosis of pleomorphic adenoma in this anatomic location should be entertained cautiously, and distinction from the many tumors that occur more frequently *(e.g.,* adenoid cystic carcinomas, mucoepidermoid carcinoma and low-grade chondrosarcoma) should be foremost in the mind of the pathologist.

Other benign tumors

Myoepitheliomas have been rarely reported in the larynx. The morphology and clinical course has been identical to their counter part in the major salivary glands.

Malignant neoplasms

Adenoid cystic carcinomas

Adenoid cystic carcinomas of the larynx comprise only about 0.25% of laryngeal carcinomas. The age range of occurrence is fairly wide, but they are found most often in the fourth to the sixth decades of life. The sex incidence is approximately equal. These laryngeal tumors most often occur in the subglottic area, but a substantial proportion are found supraglottically, Tumors of glottic origin are even less common. The histological features are the same as those seen with adenoid cystic carcinomas found at other sites. Total laryngectomy has generally been employed for treatment. Not unexpectedly, a high incidence (>50%) of local treatment failure does occur; thus postoperative radiotherapy may be beneficial. Because the reported incidence of lymph node metastasis is higher than that for adenoid cystic carcinomas elsewhere, some have recommended elective neck dissection.

Mucoepidermoid carcinoma

The presenting signs and symptoms of laryngeal mucoepidermoid carcinomas mimic those of squamous cell carcinoma of the larynx. Hoarseness is common and some patients have hemoptysis, foreign body sensation, dysphagia, or a neck mass. Reported lesions have varied in size from about 0.5-5.0 cm. Nothing in their gross appearance distinguishes them from squamous cell carcinomas. Microscopically, low-grade mucoepidermoid carcinomas of the larynx resemble the same type of tumor found in other sites, and recognition is usually not too difficult. High-grade mucoepidermoid carcinomas may resemble poorly differentiated squamous cell carcinomas. The behavior of these laryngeal tumors has been referred to as unpredictable. This may partly result from analyses that contain different numbers of high-grade adenosquamous carcinomas, which often are difficult to separate from mucoepidermoid carcinomas. Although histological grading influences treatment, the most important factor in therapy is the clinical stage. Total laryngectomy has been the most frequently employed treatment, but appropriately small or limited lesions have been treated with vertical hemilaryngectomy or supraglottic laryngectomy, In the presence of clinically enlarged neck lymph nodes, neck dissection should generally be performed. On the basic of our own experience and that of the Armed Forces Institute of Pathology, it is safe to presume that except for low-grade mucoepidermoid carcinomas, other supposed grades of that carcinoma in the larynx are much more likely to be adenosquamous carcinomas.

Adenospuamous carcinoma

This highly malignant neoplasm may arise from overlying surface mucous or from the ducts of minor salivary glands. Microscopically, adenocarcinomatous and squamous carcinomatous components should be present in a single neoplasm with intercellular bridges or keratin demonstrable in the squamous component. Approximately 40 cases have been reported in the larynx. However, the diagnostic criteria are not universally accepted, and some authors do not distinguish between adenosquamous and high-grade mucoepidermoid carcinoma. Furthermore, whether primary adenosquamous carcinoma of the minor salivary glands exists is controversial, most authors consider this carcinoma of surface origin.

Adenocarcinomas

There is also a frustrating lack of clarity in what constitutes an "adenocarcinoma" of the larynx affer exclusion of adenoid cystic carcinoma. From the literature one gets the distinct impression that the so-called adenocarcinomas are poorly differentiated, large, bulky, and preponderantly supraglottic neoplasms that are subsurface in origin. When photomicrographic illustrations are available, many, if not most, have a neuroendocrine appearance.

Miscellaneous carcinomas

A few cases of laryngeal acinic cell carcinoma have been reported in the literature. Epithelia-myoepithelial carcinoma, myoepithelial carcinoma, carcinoma expleomorphic adenoma and salivary duct carcinoma have also been reported to occur in the larynx.

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Gene alterations in precancerous and cancerous lesions of the larynx

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Stratified squamous epithelium is composed of several layers with distinct biological functions, Cells in the basal layer, which make contact with basal membrane, are stem cells with the ability to proliferate and provide new elements for the upper layers. However, the main proliferative activity is detected by Ki67 expression in cells located immediately above them, Going upwards, the prickle cell layer is the most populated, as well as the most morphologically characteristic. These cells are still metabolically active, their main function being the production of keratin. Cells that enter the prickle cell layer express p21WAF1 instead of Ki67, but this expression is transitory because it is seen only in the lower layers (1).

Appropriate mechanisms regulating cell growth and differentiation maintain the normal turnover that controls epithelial thickness, perhaps through cyclin-dependent kinase inhibitors (OKIs), such as p21WAF1. It is widely accepted that malignant transformation of squamous epithelium progresses through a number of steps, some of which can be morphologically recognized, such as the so-called

precancerous lesions (2). Alterations in oncogenes and tumor suppressor genes participate in the development of neoplastic transformation and progression.

Several approaches have allowed for the identification of the genes actually involved in these lesions, or at least, of the chromosomal regions involved. The roles of genes such as p53, p16INK4a, and p21WAF1 as tumor suppressors have been investigated in laryngeal carcinoma. The participation of oncogenes such as *ras* or cyclin D1 is also of interest. Finally, molecules involved in invasiveness, such as matrix metalloproteinases, participate in progression of carcinomas. p53 mutation is the most frequent genetic alteration identified in human cancer (3). Loss of p53 function appears as one of the limiting steps for neoplastic transformation. p53 mutations have been detected in preneoplastic lesions (4). Moreover, p53 protein overexpression, a supposed marker for p53 mutation, is a common phenomenon in premalignant laryngeal tissues (5, 6). Loss of heterozygosity (LOH) at 17p13, where p53 is located, is already detected in hyperplastic lesions, with the number of cases with LOH increasing with progression to overt dysplasia and/or carcinoma *in situ,* but no longer progressing once invasive carcinoma is developed (7). About 50% of carcinomas harbor p53 alterations, although no association is seen with stage of disease, suggesting that it does not represent any further advantage for tumor progression once initiated (1, 5).

The role of p21WAF1 as a tumor suppressor in laryngeal carcinoma was suggested by its regulation, at least in part, by wild-type but not mutant p53 (8) as well as its ability to inhibit cyclin-dependent kinase activity (9). However, in squamous cells, p21WAF1 expression occurs apparently by p53-independent pathways (as can be seen in normal squamous tissue samples). p21WAF1 expression increases in preneoplastic lesions and can be detected throughout the entire thickness of the epithelium. Most of carcinomas express high levels of both mRNA and protein. Only poorly differentiated carcinomas have low mRNA levels and appear negative by immunohistochemistry. Even within moderately differentiated carcinomas, areas with better squamous differentiation are more strongly positive than less differentiated areas (1). Use of p21WAF1 for gene therapy in squamous cell carcinoma of the head and neck, however, has so far proven unsuccessful (10).

p16INK4a is the first member of a family of CKIs with known tumor suppressor activity (11). It is located at 9p21, another chromosome region that is frequently deleted in hyperplasia and more frequently in carcinoma *in situ,* suggesting a progression in preinvasive lesions. The frequency of LOH at 9p21 is the same in both carcinoma *in situ* and invasive carcinoma, similar to what is seen for p53 (7). There are several ways of inactivating p16INK4a, including mutation or promoter hypermethylation associated with LOH or homozygous deletion (12, 13), all of which are found in a number of carcinomas (14), but the finding of LOH in preinvasive lesions suggests that inactivation of p16INK4a can precede invasiveness. However, the INK4a locus has a complex structure, coding for two different transcripts, both with tumor suppressor activity, but through different pathways (15). In fact, p19ARF (p14ARF in humans), exerts its tumor suppressor activity through sequestering and promoting degradation of MDM2, a cellular protein that binds to, and promotes degradation of, p53. Therefore, the exact role of the two genes in tumor suppression by INK4a locus is still unknown.

Some other tumor suppressor genes are likely to participate in laryngeal carcinogenesis. These are known to be at 3p21 or nearby, although they are still far from fully characterized. Activation of proto-oncogenes to oncogenes is the other main feature present in neoplastic transformation. Although theoretically many genes could be involved in squamous cell transformation, very little is known about the actual *in vivo* role of most of the oncogenes. An important event in many malignancies is the participation of the ras family, which represent the largest number of oncogenic alterations in human cancers. However, ras activation (usually by point mutation) appears as a key event in glandular, but not squamous transformation. Most of reports agree that there is an extreme rarity of ras mutations in head and neck cancer (16), although Ras protein overexpression is a common finding in carcinomas, independent of gene mutation, which, surprisingly, appears associated to a favorable patient outcome (17). On the other hand, the participation of cyclin D1 in laryngeal carcinomas has been well established. Cyclin D1 is the regulatory subunit that activates cyclin-CDK complexes, and it is associated mainly with CDK4 (18). Amplification leading to overexpression of cyclin D1 is associated with tumor progression in invasive carcinomas (19). Whether this is due to higher aggressiveness of the tumors or because it is a late event in neoplastic progression remains unexplained.

The key feature that differentiates preinvasive and invasive lesions is the progression through basal membrane. There, neoplastic cells progress according to their ability to detach from surrounding cells, to attach to stromal elements, promote degradation of stromal macromolecules and move on. All these features are necessary and must be tightly coordinated to be effective. Loss of adhesion molecules has been reported as well as analysis of different proteolytic enzymes. Most of the enzymes show an increase in its production or activity in tumors when compared to non-neoplastic tissues. However, production of some enzymes appears specific to neoplastic tissue. This is the case of collagenase-3, a member of the matrix metalloproteinase family (20) that is detected in invasive tumors but not in normal laryngeal mucosa. Moreover, expression of collagenase-3 is limited to advanced tumors, suggesting that these tumors have an invasive advantage. Collagenase-3 is activated by proteolysis of procollagenase-3. This is efficiently achieved by other members of the family, gelatinase A and the membrane-type matrix metalloproteinase, which are coordinately overexpressed in cases with collagenase-3 expression (16). In summary, loss of tumor suppressor function is implicated in the first steps of malignant transformation, although it does not confer additional advantages to invasive tumors. Activation of oncogenes participates in later steps, near the acquisition of the invasive phenotype, where a collaboration with rearrangement of adhesion molecule expression and modulation of proteolytic enzyme production is needed for further tumor progression.

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Squamous papilloma of the larynx

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Papilloma is a benign exophytic neoplasm of epithelium on a connective tissue core. In the larynx the stratified squamous variety is the commonest form of papilloma. They are found in both adults and children, but, in the latter, because of the much narrower diameter of the airway, the symptoms are more serious and treatment is more urgent and difficult. It is thus customary to divide the condition on the basis of the age of the patient into juvenile and adult types. In some juvenile cases the papillomas persist into adult life. The histopathological appearances are similar at all ages. By far the commonest site of occurrence of squamous papilloma in the larynx is the vocal cord, usually in the anterior half. Multiple papillomas may, particularly in children, spread upwards to the supraglottis, pharynx and soft palate. Squamous papillomas range from white to red, and are delicate, granular, polypoid structures which vary from 1-10 mm in diameter, most being less than 5 mm. In florid cases the papillomas form a solid field of mucosal thickening without invasion deep to mucosa. Under magnification, small individual papillae can be discerned as blunt finger-like processes with branches, which never become long and filiform. Microscopically, the papillary processes are seen as cylindrical projections with smaller offshoots of squamous-cell-covered epithelium cut in various planes, being second or even third order branching of the papillary structures. In a minority of Cases there is keratosis in which layers of completely keratinized anucleate cells are seen on the surface of the papillae.

Sometimes cells of the squamous epithelial covering of the papillae show atypical change, a situation which is frequently related to the presence of koilocytosis. Epithelial atypia has been alleged to be associated with rapid recurrence of papillomas and increased risk of progression to carcinoma. Koilocytosis is frequently seen in the upper, intermediate and superficial zones of the squamous epithelium of laryngeal papillomas. It consists of a spherical enlargement of the cells of the lesion, accompanied by perinuclear vacuolation, so that either no stained cytoplasm is seen in the cell at all, or there is but a thin rim of cytoplasm around the cell periphery. The nucleus is central and often enlarged, angular or wrinkled. It may exhibit moderate degrees of atypical change. Infection of the cells of squamous papillomas of the larynx by human papillomavirus is frequent (see below), and has been closely correlated with the presence of koilocytosis. In a few cases of papillomatosis some of the material shows a papillary transformation not only of squamous cell epithelium, but also of respiratory epithelium. The latter comprises nonmalignant respiratory epithelium featuring both ciliated cells and goblet cells, the appearances being reminiscent of those of the entity of cylindric cell papilloma of the nose and paranasal sinuses. Papillomas showing respiratory epithelial hyperplasia have a decided tendency to recur. Difficulty may be experienced in distinguishing two other types of neoplastic lesion, which occur particularly often on the vocal Cords, from squamous papilloma: i) keratotic plaque with dysplasia; and ii) carcinoma of both verrucous squamous and regular types.

Raised plaques composed of thickened squamous epithelium with a keratinized surface are seen quite frequently in biopsy. They show a mild to severe degree of dysplasia of the deeper layers of their squamous cells. It is important to separate these lesions from squamous papillomas because the former have a malignant potential which is not possessed by the latter. A careful examination of the whole biopsy for evidence of cylinders of papilloma formation and the branching that is associated with them will usually suffice to distinguish this lesion. The degree of dysplasia exhibited by the plaque is often more severe than would be expected in a papilloma.

Squamous carcinoma may exhibit papilloma formation and should not be mistaken for benign squamous papilloma. The pattern is not, however, as symmetrical as in papillomas and branching of papillae is unusual. Verrucous squamous carcinoma may display very long papillae, usually without branching. The rete ridges are irregular and the basement membrane is rarely hyalinized, a feature which is frequent in papillomas. Squamous cells of the intermediate layer in verrucous carcinomas and some papillary areas of regular squamous carcinoma are larger than those of the corresponding layer in squamous papillomas, showing a mean area of more than 300 æm^2 on image analysis.

Squamous papillomas arising in childhood, although similar in morphology and etiology to those arising in adults are distinct in some important respects. Their incidence has a female preponderance, unlike those occurring in adults, which have a male preponderance (see below). They may arise at any age of childhood and have been described as early as 18 months. Treatment in juvenile onset cases is more difficult and more prolonged than the adult onset papillomas and children, having smaller airways, require tracheostomy more frequently than do adults. After tracheostomy the tendency of papillomas to spread down the trachea to the main bronchi is greater in children than in adults. After many episodes of surgical removal followed by recurrence, the florid papillomas may eventually cease to recur. Malignant alteration of juvenile papillomatosis has been described. A few cases are on record in which malignancy took place many years after treatment of juvenile papillomatosis by radiotherapy. In these cases, it must be presumed that the carcinoma was the result of the radiation.

Most adult papillomas arise *de* novo in adult life with an age incidence which is maximum in the fourth decade. About 20% of adult papillomas have arisen in childhood. Males are affected twice as often as females. The maximum incidence of onset is in the fourth decade. The tendency to recurrence, and hence the number of operations required for removal of adult papillomas, is related to the form which the papillomas take at the onset. If the papillomas form a solitary mass in the larynx there is little tendency to recurrence and a single endoscopic operation to remove the papilloma is required. Two or more distinct laryngeal lesions, separated by clinically normal epithelium, require many more endoscopic operations. A third group which may be designated as florid papillomatosis, where there is involvement of every part of the larynx, necessitates frequent removals, eventually tracheostomy and even laryngectomy.

A viral basis for squamous papillomas of the larynx has been under consideration for many years. Viruses leading to neoplasia may be classified as either RNA viruses, such as the virus which produces the Friend virus lymphoma in mice, or DNA viruses, such as that producing the Shope papilloma of rabbit skin and the Papova virus. The human papillomavirus (HPV) is a member of this latter group which is known to give rise to the lesions of verruca vulgaris and condyloma acuminatum. It seems likely that squamous papillomas of the larynx are also caused by this virus. The HPV has not been propagated in culture. Electron microscopic observations have for the most part been unsuccessful in the search for virus particles in laryngeal papillomas. Virus-like structures have been found in the nuclei of laryngeal squamous papilloma cells after an extensive search, however. The localization of HPV antigen using the immunoperoxidase method in paraffin sections of squamous papillomas has yielded more encouraging results. The antibody detects HPV antigen in nuclei of squamous cells of the papillomas, near the surface of the tumor. Greater sensitivity in the detection of HPV and its types has become available with the use of molecular biological techniques, in particular that of *in situ* hybridization which has enabled the demonstration of HPV type 6 and the related type 11 in the cells of benign papillomas, The hybridization signal, like that of immunocytochemical positivity, is always confined to the nuclei of the superficial stratified squamous epithelium. Both viral types are usually found together. About 66% of both adult and juvenile cases are positive for HPV types 6 and 11. The proportion of cases giving positive signals for HPV is, however, much higher in cases with multiple confluent lesions than in those with single isolated lesions. It seems possible that the detection of HPV 6 and 11 in biopsy specimens at initial endoscopy might be a useful prognostic indicator, because those patients whose biopsy tissue shows many nuclei positive for HPV types 6 and 11, especially on more than one occasion, are more likely to have a worse eventual outcome. The presence of HPV 6 and 11 may also be used to monitor interferon treatment. The presence of signal for virus is closely correlated with that of koilocytosis in routinely stained sections.

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