Carcinoma of the head and neck in the HPV era

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head and neck, carcinoma, HPV, histological variants

ABSTRACT

This review encompasses the most salient advances in the understanding of the biopathology of head and neck squamous-cell carcinoma (HNSCC) accomplished over the last decade, emphasizing the significant role played by high-risk HPV genotypes. This has led to a new and meaningful subdivision of conventional HNSCC in two main prognostic and therapeutic groups: 1) keratinizing HNSCC, mainly occurring in elderly men that are heavy smokers and drinkers, TP53 mutated and/or p53-positive, HPV16-negative, being associated with an aggressive course; and 2) non-keratinizing HNSCC, occurring in younger men between 40 and 60 years that are non-smokers and non-drinkers, HPV16- positive, p16-positive, and p53- negative, being associated with improved prognosis. The main risk factors are number of sexual partners, oral-genital sex, oral-anal sex, and marijuana use. Among the unusual variants of HNSCC, papillary and lymphoepithelial-like are mostly related to HPV-16 infection, whereas the spindle and acantholytic types are mainly associated with tobacco and alcohol. The basaloid, adenosquamous, and verrucous variants may be related to both types of risk factors. Spindle cell carcinoma has been shown to be a prototype of epithelial mesenchymal transition. The hallmark of the novel and aggressive entity "undifferentiated midline carcinoma" is the rearrangement of the Nuclear Protein in Testis (NUT) gene at t[15; 19]. In the HPV era we are proposing the Ljubljana Classification (LC) as the recommended system for grading precursor lesions in heavy cigarette smokers and alcohol drinkers and the dysplasia and SIN systems for grading intraepithelial precursor lesions related to the increasingly detected epidemic of HNSCC associated with high-risk HPV infections.

Introduction

Head and neck cancer is one of the most common cancers worldwide, More than 90% of these tumors are squamous-cell carcinomas (SCC), accounting for 5 to 10% of all new cancer cases in Europe and the U.S. (1). Throughout the twentieth century this large set of head and neck SCC (HNSCC) was clinically considered to be a rather uniform group; their worldwide variations in incidence and anatomic distribution were overwhelmingly attributed to demographic differences in the habits of exposure to smoking or chewing

tobacco and drinking alcohol (2, 3). With the advent of the twenty-first century this view has evolved because refinements of the molecular gene-technique are allowing for the recognition of new subtypes of HNSCC that differ not only in etiology, but also in pathogenesis and clinical outcome (4–6). This paper reviews the most salient advances in the pathology of HNSCC accomplished by the early twenty-first century, emphasizing the increasing role of high-risk HPV genotypes.

Conventional HNSCC

Up to 10 histological types of HNSCC were recognized by the WHO classification of head and neck tumors in 2005 (3) and about 90% of these carcinomas were lumped together within the category of conventional HNSCC. Although anatomic location, extension, and depth of invasion influenced the prognosis of these tumors, their predictive value was rather limited. Also limited was the prognostic value of grading conventional HNSCC in well-, moderate-, and poorly-differentiated subtypes, either keratinizing or non-keratinizing varieties, as well as in exophytic or endophytic (3). Nevertheless, shortly after this WHO publication was released (3), a new and meaningful subdivision of conventional HNSCC started to gain acceptance. Based on the combination of epidemiology, histology, immunohistochemistry, and molecular genetics, the two prognostic and therapeutic types given below were delineated.

Keratinizing HNSCC p53-positive and HPV16-negative, in smokers and drinkers

The patient paradigm for this type of HNSCC is a man over 60 years old that smoked cigarettes and drank alcohol for many years (7). Histologically, keratinization of squamous epithelial cells with variable "pearl" formation and invasive growth are the prerequisite features of this type of tumor. Well-differentiated SCC contains large keratinocytes that resemble normal squamous epithelium and produce abundant keratinization. Moderately-differentiated SCC usually shows less keratinization and distinct nuclear pleomorphism, with mitotic activity and abnormal mitoses. In poorly-differentiated SCC immature cells predominate, with numerous atypical mitoses and minimal but patent keratinization (8). Although keratinization is more likely to be present in well- or moderately-differentiated SCC, these features should not be considered a significant prognostic criterion because most keratinizing SCC are moderately differentiated. Grading by pattern of invasion bears more discriminative prognostic value. The expansive growth pattern is characterized by large tumor islands with well-defined pushing margins and is associated with better prognoses. The infiltrative growth pattern is characterized by scattered small cords or single tumor cells with poorly-defined jagged margins and is associated with a more aggressive course (8). More often than not, keratinizing SCC shows an infiltrative growth pattern, although a mixed expansive-infiltrative pattern can also be seen. These tumors are likely to metastasize to regional lymph nodes. Preoperative accurate assessment of lymph node status can be significantly improved through cDNA microarray studies, leading to a remarkable increase in the percentage of patients receiving the appropriate treatment (9).

Common to all the keratinizing HNSCC is the field cancerization effect that may cause synchronic and metachronic second SCC. The modern concept of field cancerization implies that genetically modified basal epithelial cells initiate a phase of horizontal growth, along the basal and parabasal layers, forming first a localized "patch," expanding later to a "field" that eventually progresses to carcinoma (10). Of the mechanisms involved in the pathogenesis and progression of keratinizing HNSCC, those that govern the transition of the cell-cycle from G1 to S phase have been studied in more detail. Among them, alterations of p53, p16, CDK4, and cyclin D1 are quite relevant, whereas alterations of retinoblastoma (Rb) gene expression seem to be an infrequent event (11). TP53 gene mutations have been reported in up to 50% of HNSCC (12) and p53 overexpression in about 65% (13). Mutations are mostly found at exons 5–9 and the transversions at codons 157, 173, and 273 are considered characteristic of exposure to tobacco smoke. The TP53 mutational pattern in laryngeal SCC seems to be more similar to carcinoma of the lung than to other HNSCC (14). Out of 13 laryngeal carcinomas with TP53 mutations, six missense, one nonsense, and six frameshift mutations were observed. G to T transversions predominated among the non-frameshift mutations (15). One homologue of p53 is the gene p40 and its isoforms p51, p63, and p73L, localized distally at 3q. The main difference between the various transcripts is the presence or absence of the transcriptional activation domain TA. Transcripts lacking the TA domain, like p40, play an oncogenic rather than suppressive role. Amplification of this gene was found in up to 60% of HNSCC and of SCC of lung, hence the acronym AIS ("amplified in squamous cancer"). AIS amplification may result in a p53 independent pathway of SCC transformation (16, 17). A marked reduction of the protein expression of the fragile histidine triad suppressor gene FHIT, localized at 3p, has been observed in HNSCC and its

precursors (18) and in SCC of the lung preferentially in smokers (19).

Inactivation of CDKN2A is a relevant alteration in keratinizing HNSCC by deregulating the catalytic activity of CDK4/cyclin D1 complexes that induce the cell-cycle to progress. Genetic alterations of CDKN2A were found in 57% of laryngeal carcinomas (20, 21). Of these, 27% were mutations, 23% homozygous deletions, and 7% hypermethylations. CDKN2A codifies two transcripts $p14^{ARF}$ and $p16^{INK4a}$. Loss of heterozygosity at 9p21–23, the locus where CDKN2A is located, strongly correlates with cyclin D1 overexpression in advanced stage HNSCC (20). Cyclin D1 amplification was found in 37% of tumors and overexpression in 35%. This association was particularly relevant in advanced-stage tumors, where gene amplification and mRNA overexpression occurred in 60% of cases (22). Overexpression of CDK4, the main partner of cyclin D1 in the G1-phase of the cell cycle, was reported in 70% of laryngeal carcinomas. All but one of the tumors expressing high levels of cyclin D1 also overexpressed CDK4. This overexpression occurred at the transcriptional level, without gene amplification (23). Cooperation between all the previously discussed gene-products seems to be relevant not only to the progression but also to the therapy of these HNSCC. Association of cyclin D1 amplification and of TP53 mutations correlates with resistance to treatment with cisplatin (24).

The eukaryotic translation initiation factor 4E (eIF4E) was reported to be overexpressed in 100% of laryngeal carcinomas and in 59% of surgical margins (25). Because activation of the Akt/mTOR pathway and dysregulation of PTEN results in activation of eIF4E, this pathway opens the possibility of using rapamycin analogues, which are mTOR inhibitors, as adjuvant therapy for residual disease in laryngeal carcinoma (26). One recent therapeutic target in HNSCC is the EGFR pathway because this receptor is overexpressed in 70% of laryngeal SCC. Various monoclonal antibodies (mAb) can inhibit the activity of EGFR, inducing apoptosis and chemo- and radio-sensitization in advanced-stage tumors; this inhibition may have additional effects because EGFR also up-regulates cyclins and proteases (27). Matrix metaloproteinases (MMP) are overexpressed in laryngeal SCC, among them gelatinase A (MMP-2), collagenase-3 (MMP-13), and membrane-type-1 MMP (28), the latter two particularly at advanced stages; they are related to invasion and may be involved in chemotherapy resistance. Blocking VEGF and its receptor VEGFR by mAb is another recently proposed therapy (29). This may be of additional value when combined with antibodies against EGFR because the simultaneous overexpression of both factors decreases survival in SCC (30). iRNA technology was shown to knockdown Stat3 expression in laryngeal carcinomas transplanted to nude mice, inhibiting tumor growth and inducing apoptosis (31). Other approaches are currently under investigation to further explore new specific therapies.

Non-keratinizing HNSCC HPV16-positive and p53-negative, in non–tobacco smokers and non-drinkers

Non-keratinizing HPV-positive cancers are increasingly recognized as a subgroup of HNSCC with a distinct clinical, histological, and biological profile (4). The paradigm of a patient with this tumor is a younger man between 40 and 60 years old that has never smoked cigarettes or drunk alcohol (32, 33). Microscopically, islands of squamous epithelial cells with absence of keratinization and invasive growth are the prerequisite features of this type of tumor. Typically, there is lack of maturation in the epithelial squamous cells and a moderate to significant degree of atypia, which may provide a basaloid appearance. The tumor is usually moderately or poorly differentiated; when it is poorly differentiated it is difficult to recognize as SCC. Occasionally, some degree of keratinization may be seen. When keratinization is conspicuous, there may be microscopic overlap with keratinizing HNSCC (34). Some of these tumors may also overlap with the papillary, basaloid, and lymphoepithelial-like subtypes of HNSCC. Non-keratinizing SCC invades the underlying tissue with an expanding, smooth, lobulated, and generally well-delineated border, although foci of infiltration by irregular small nests or strands may be seen. Association with squamous dysplasia has been observed in the crypts of the tonsils, where the specialized tonsillar squamous epithelium is normally found. Extension of dysplastic changes to the surface epithelium is a very rare observation, which is seen together with involvement of the crypts; no field cancerization effect is seen (35). Metastasis of this tumor to the regional lymph nodes may show very conspicuous cystic degeneration (36).

Non-keratinizing HNSCC HPV-positive mainly occurs in the oropharynx, where HPV16 is detected in 70% of tumors (4, 37). In the sinonasal tract HPV16 has been found in 20% of SCC (38, 39). Much less frequently, HPV16 is detected in carcinomas of other regions such as the larynx (40), hypopharynx (41), and nasopharynx (42). In all these tumors, other less common types of high-risk HPV may be occasionally detected (39, 40, 41). Although the best method for HPV detection remains controversial, a commonly accepted strategy has been the use of PCR and SPF-10 primers (39). More recently, the combination of p16 im-

munohistochemistry followed by in-situ hybridization (ISH) for high-risk HPV has been proposed as more reliable than PCR-based methods (4). Very recently, p16 immunohistochemistry alone has been advocated as the best test to predict outcomes in patients with HNSCC (43) because its strong and diffuse pattern of p16 immunostaining serves as a highly sensitive surrogate marker for identifying HPV-positive tumors. In HPV tumorigenesis, p16 acts as a tumor suppressor protein that inhibits CDK4, preventing in turn the transition of the cell-cycle from G1 to S phase (44). In SCC with biologically active HPV, inactivation of the Rb protein by the HPV E7 protein leads to p16 overexpression because Rb normally represses the transcription of p16. HPV-positive HNSCC also expresses the oncoprotein E6 that binds and degrades wild-type p53 protein. Unlike carcinomas of the uterine cervix, in which HPV infection and p53 mutations are mutually exclusive events, HPV infection and TP53 mutations sometimes occur together in HNSCC (45), but disruptive TP53 gene mutations are not encountered in HPV-positive carcinomas (46).

The clinical behavior of HPV-positive HNSCC is associated with encouraging prognoses (5, 38, 47, 48). The mechanisms underlying this favorable outcome may involve the combined effects of immune surveillance to viral specific tumor antigens, an intact apoptotic response to radiation, and the absence of widespread genetic alterations associated with smoking (4, 49, 50). Epidemiological factors point to sexual practices, the most relevant of these being the number of sexual partners and history of oral-genital sex and oral-anal sex (33, 51). Marijuana use has been recently identified as an independent risk factor for HPV+ HNSCC (52) because marijuana smoke modifies antitumor immune response by binding cannabinoids to the CB2 receptor expressed in human tonsillar tissue. HIV-infected patients have a higher incidence of HNSCC than non-HIV-infected patients; the HPV-associated tumors of the former group arise from sites similar to those of non-HIV-infected patients, such as oropharynx and sinonasal tract (53). In this type of population laryngeal SCC is highly associated with alcohol abuse and tobacco smoking (54).

Variants of HNSCC

This category, although encompassing about 90% of the histological subtypes of HNSCC, accounts for only about 10% of these tumors (3). Until recently their rarity compared to conventional SCC has hampered progress in understanding the biological features and pathogenetic mechanisms underlying the diversity of phenotypes and clinical features of these neoplasms.

Verrucous Carcinoma

Verrucous carcinoma (VC) accounts for about 3% of all HNSCC; it is a very well-differentiated SCC that occurs predominantly in men in their 50s and 60s. It is characterized by an exophytic warty growth, which is slow but locally invasive, and it can cause extensive local destruction if left untreated, but rarely if ever metastasizes (55). Remarkably, 75% of VC arises from the oral cavity and 15% from the larynx (56). In the oral cavity, the sites most frequently involved are the buccal mucosa and the gingiva, and in the larynx the vocal cords are the most common site of origin (55, 57, 58). VC rarely occurs in other locations of the head and neck (59).

Chewing tobacco mixtures has been implicated in the high incidence of VC of the oral cavity in India (60). In addition to tobacco-related carcinogens, another etiologic factor could be HPV because HPV types 16 and 18, and rarely types 6 and 11, have been found in some but not all cases of VC (61, 62, 63). Microscopically, church-spire keratosis on the surface, thickened club-shaped intrastromal invaginations at the base, misleading lack of cellular atypia, and locally invasive blunt pushing margins are the main distinguishing histological features of VC (64). A lymphoplasmacytic inflammatory response is common in the stroma. The keratinocytes of VC are usually larger than those of conventional SCC and lack the usual cytological criteria of malignancy (65). They express TGFβ-R (66). Mitoses are rare and only observed in the suprabasal layer; there are no abnormal mitoses.

Hybrid (mixed) VC (HVC) is composed of VC admixed with foci of conventional well-differentiated SCC (67). In most instances, this latter conventional SCC component is well-differentiated, always arises at the base of the tumor, and is not frequently is difficult to find; thorough sampling of all VC is therefore mandatory to rule out HVC (67). The reported incidence of HVC in the oral cavity is 20% (68) and in the larynx 10 % (58). HVC bears potential for metastasis to regional lymph nodes. Patients with HVC must be treated aggressively as if they had conventional keratinizing SCC (58, 68). HVC has recently been proposed as the most plausible explanation for the controversial cancer that afflicted German Emperor Frederick III (69).

Papillary SCC

Papillary SCC (PSCC) is an uncommon variant of HNSCC that occurs predominantly in males in their 50s and 60s, characterized by an exophytic, papillary growth pattern and a good prognosis (70). PSCC shows predilection for the oropharynx, hypopharynx, larynx, and sinonasal tract (71, 72, 73, 74, 75). The main microscopic feature of PSCC is the papillary

growth pattern, which must comprise the majority of the tumor mass (76). Papillae are made up a central fibrovascular core covered by neoplastic squamous epithelium of varying thickness. The covering epithelium may be composed of immature basaloid cells, or may be more pleomorphic resembling carcinoma in situ. Mitotic figures are abundant. PSCC is usually non-keratinizing or minimally keratinizing. A keratinizing form of PSSC that never exhibits as much hyperkeratosis as in VC is recognized. Multiple lesions can be found in some patients, consisting of either invasive PSCC or mucosal atypical hyperplasia. PSCC may show koilocytic features (76). The tendency to invade appears late. If no stromal invasion is found the tumor should be called PSCC in situ or non-invasive PSCC (77). Although metastases to regional lymph nodes can occur in PSCC, their prognosis is better than that of conventional SCC.

As in conventional SCC, smoking and alcohol have been assumed to be an important etiological factor in PSCC (71, 76). Because of clinical and histopathological similarities between PSCC and squamous-cell papilloma, it was postulated that HPV might be an important etiologic factor in PSCC (74). This postulate has proved to be correct because new techniques have quite recently demonstrated the frequent association of non-keratinizing PSCC with high-risk HPV-16 in the sinonasal tract, oropharynx, and larynx (37, 38, 39, 40). These HPV-positive PSCC constantly show strong and diffuse positive immunostaining for p16, and patients with these tumors have a better prognosis than their p16-negative counterparts (40, 43). Strong nuclear immunoreactivity for p53 is not an infrequent finding in PSCC (70).

Spindle Cell Carcinoma

Spindle cell carcinoma (SPCC) is a biphasic monoclonal tumor with divergent differentiation, composed of SCC either in situ and/or invasive and a malignant spindle component with a mesenchymal appearance but of epithelial origin (78). SPCC occurs predominantly in males in their 60s (89, 90). It has been linked to smoking cigarettes and drinking alcohol, and may develop after radiation exposure (79, 80). In the head and neck SPCC occurs most frequently in the larynx and oral cavity, followed by the tonsils, sinonasal tract, and pharynx (81). Grossly, it usually exhibits a polypoid appearance of variable size and less frequently may appear as an ulcerative-infiltrative lesion. Microscopically, when both malignant components, squamous and spindle, are full-blown, the diagnosis of SPCC is straightforward. Not infrequently, the squamous component of SPCC is rather inconspicuous, appearing as a deceivingly benign covering on the surface or as bland squamous nests within the tumor. In addition, there are instances in which the spindle-cell carcinoma appears almost exclusively, composed of spindle cells mimicking a sarcoma (78). There is mounting molecular evidence that SPCC is an epithelial monoclonal neoplasm with divergent monoclonal (mesenchymal) differentiation (82–84). In such cases, cytokeratins are demonstrated in 40 to 85% of SPCC; the more cytokeratins are used, the higher the chances of a positive reaction. Although controversial, limited immunoreactivity for polyclonal cytokeratins has been associated with significantly improved survival rates (85, 86).

In 2008, Zidar et al. reported that the expression of E-cadherin disappears in the spindle cell component of SPCC, whereas N-cadherin, a marker of mesenchymalization, is neo-expressed in the spindle cells but not in the squamous cell component. Moreover, expression of catenins was altered and Snail-1 expression, a potent inducer of epithelial mesenchymal transition (EMT), was found in more than half of cases (87). A further study demonstrated the up-regulation of mRNA of transcription factors Snail, Slug, Twist, and SIP1 in SPCC when compared to SCC. All four factors are triggers of EMT. Immunohistochemistry demonstrated a positive reaction for Slug and SIP1 in all cases and for Snail in two thirds of SPCC cases (88). More recently, the down-regulation of microRNAs of the miR-200 family and miR-205, and an altered expression of classical and desmosomal cadherins has been proposed as a hallmark of EMT in SPCC of the head and neck (89).

Basaloid Squamous Carcinoma

Basaloid squamous-cell carcinoma (BSCC) is an aggressive, high grade, biphasic variant of SCC with basaloid and squamous components (90). BSCC occurs predominantly in men 60 to 80 years old (91–94). Pyriform sinus, base of tongue, and supraglottic larynx are the most frequent sites in the upper aerodigestive tract (91, 92, 95–97). An advanced stage is usually present at the time of diagnosis. Metastases to regional lymph nodes are seen in two-thirds of patients (93, 98–100). Microscopically, BSCC are composed of small, closely packed basaloid cells, with hyperchromatic nuclei, with or without nucleoli, and scant cytoplasm. The tumor grows in a solid pattern, with a lobular configuration and with a frequent peripheral palisade of the nuclei. Central keratin pearl formation and large comedo-type necrosis are frequent findings. In addition, abundant intercellular hyaline globules are found, conferring a cribriform-like pattern whose differential diagnosis must include adenoid cystic carcinoma. BSCC is always associated with a squamous

component, which may be present either as an in-situ or invasive SCC (91, 92, 94). Although tobacco and alcohol abuse have been proved to be strong risk factors for BSCC (93, 94, 98, 99), a few years ago the detection of HPV16 by in-situ hybridization was reported in 34% of tumors out of a series of 53 BSCC of the head and neck. Of them, 16 of 21 (76%) HPV16-positive BSCC originated in the oropharynx. Only 2 of 32 (6%) HPV16-positive BSCC arose in non-oropharyngeal sites. The absence of HPV16 was significantly associated with decreased overall survival, even though patients with HPV-positive BSCC were more likely to present with lymph nodes metastases (41).

Adenosquamous carcinoma

Adenosquamous carcinoma (ADSC) is a rare aggressive neoplasm that originates from the surface squamous epithelium and is characterized by both SCC and adenocarcinoma (101). ADSC has a male predisposition with a tendency to develop when patients are in their 50s and 60s. Cigarette smoking and alcohol consumption have been implicated (102). The role of gastroesophageal reflux has not been well established clinically, but it has been well demonstrated experimentally (103, 104, 105). Furthermore, in a very recent series of 18 ADSC, three cases (16%) showed HPV E6 and E7 and p16 expression, one from the nasal cavity and two from the oropharynx (106). The most frequent site of origin for ADSC in the head and neck is the larynx, followed by the nose and paranasal sinuses and by the oral cavity (102, 107–109). Microscopically, ADSC is a biphasic tumor with squamous and glandular malignant components. Both originate from squamous epithelium. SCC presents either in situ or invasively and adenocarcinoma is seen at the lower invasive parts of ADSC, exhibiting formation of malignant glands (101, 107). Metastases to regional lymph nodes occur in three-fourths of the patients and distant metastases in one-fourth. The biphasic pattern of ADSC is retained in lymph node metastases (110). The main differential diagnosis of ADSC is high-grade mucoepidermoid carcininoma (MEC); the correct distinction of both entities is of importance because this MEC bears a better prognosis than ADSC. In ADSC, the presence of SCC in situ appears in continuity with malignant glands, a pattern that is never seen in MEC (101). In cases of secondary invasion of the squamous epithelium by high-grade MEC, malignant glands are constantly encountered in continuity with either normal or hyperplastic squamous epithelium, a finding that is never seen in ADSC. Moreover, in 2005 Alos et al. reported a strong expression of the membrane-bound mucin MUC1 in high-grade MEC, an immunoreaction that was not observed in ADSC (111); this finding is of use in the differential diagnosis between the two types of tumor.

Acantholytic squamous-cell carcinoma,

Acantholytic squamous-cell carcinoma (ASCC), also known as adenoid SCC or angiosarcoma-like SCC, is characterized by marked acantholysis of the tumor cells creating pseudolumina and a false appearance of glandular differentiation. There is no evidence of true glandular differentiation or mucine production (112). In the head and neck it arises most frequently in the skin, especially in sun-exposed areas (113, 114). No particular etiological factor has been discovered for the mucosal ASCC (115). In the mucosal sites ASCC arises in the lip, oral cavity, larynx, and hypopharynx (116). ASCC are composed of islands and cords of keratinizing SCC; the acantholysis of neoplastic cells gives rise to pseudoglandular structures that have central lumina containing acantholytic neoplastic cells, necrotic debris, or they may be empty. The conventional SCC component is nearly always present. Acantholysis may lead to the formation of anastomosing spaces and channels, thus mimicking an angiosarcoma. Prognosis of ASCC is similar to conventional SCC, which means better outcome than for ADSC and angiosarcoma. ASCC is positive for CK5, like conventional SCC. Contrary to ADSC, it is negative for CK7, a marker of glandular epithelium in the upper aerodigestive tract. Opposite to angiosarcoma ASCC is negative for CD31. Due to the rarity of this neoplasm, the molecular mechanisms triggering the acantholysis have not been well established.

Lymphoepithelial-like carcinoma

Lymphoepithelial-like carcinoma (LELC) is an undifferentiated carcinoma with a prominent, reactive lymphoplasmacytic infiltrate morphologically indistinguishable from nasopharyngeal carcinoma (NPC) (117). Microscopically, LELC shows a rather prominent SCC component in about half of the cases; carcinoma in situ may be seen. Sometimes, LELC may exhibit such a dense lymphoplasmacytic infiltrate that it mimics malignant lymphoma. LELC are aggressive tumors with a propensity for regional lymph node and distant metastases. Epstein-Barr (EBV) virus is uncommonly demonstrated (118, 119, 120, 121). Almost all reported cases of LELC have occurred in Caucasians (118, 119). These tumors preferentially occur in the larynx, hypopharynx, and oropharynx (119, 120, 122). Recently, 22 oropharyngeal LECL have been reported to be p16-positive by immunohistochemistry, as well as HPV16- positive and EBV-negative by ISH (122).

NUT undifferentiated midline carcinoma

NUT undifferentiated midline carcinoma (NMC) is a recently described aggressive carcinoma that mainly involves the head and neck; it occurs in children, young adults, and also in older patients, characteristically associated with chromosomal rearrangement of the Nuclear Protein in Testis (NUT) gene (6). In two-thirds of cases NUT is fused to BRD4, giving rise to the translocation [15;19], resulting in the NUT-BRD4 oncogene (123, 124). The effect on the NUT protein is unknown. BRD4 has a role in transcriptional activation and segregation of HPV during mitosis (125). In the remaining NUT-variant cases the fusion partner is unknown. Histologically, NMC are totally undifferentiated or may have focal squamous differentiation. Immunohistochemistry using NUT antibody followed by FISH with probes for the 15q14 NUT break-point may be extremely helpful in the identification of these tumors (126). CD 34 (6) and p63 (135) are not infrequently expressed in these tumors. The survival of patients with NMC is typically less than one year (127, 128).

Precursor and related lesions of HNSCC in the HPV era

As proposed by the WHO in 1991, the dysplasia grading system for precursor and related lesions of HNSCC was a scheme extrapolated from the uterine cervix to classify the different steps of progression of the squamous intraepithelial lesions at risk of developing into invasive squamous-cell carcinoma (129). This scheme was thought to be applicable to all head and neck regions covered by squamous epithelium, regardless of their potential variations in exposure to carcinogenic factors and in pathogenesis. Nevertheless, a change in this approach was already initiated in the WHO classification of head and neck tumors of 2005, where three different grading systems were proposed and compared, the dysplasia system, the squamous intraepithelial neoplasia system, and the Ljubljana grading system (130).

In 1995, the seminal book *Epithelial Hyperplastic Lesions of the Larynx* by Kambič and Gale (131) set out the Ljubljana Classification (LC). Ever since then, the LC has progressively gained in acceptance, being recognized in 2005 by the WHO (130) and presented as the most reliable standard for grading precursors of HNSCC (132, 133). The histopathological features of the LC have been well documented (134, 135) and are categorized as follows: simple hyperplasia (SH), basal-parabasal cell hyperplasia (BPCH), atypical hyperplasia (AH), and carcinoma in situ (CIS).

SH shows a single layer of basal cells and an increased number of the layers of keratinocytes. BPCH is characterized by piling-up of benign basal cells at the parabasal and higher layers. AH "truly risky epithelium" shows significant nuclear atypia of keratinocytes at different layers. CIS is characterized by marked cytological atypia in the practical full thickness of the squamous epithelium. At the molecular level, the studies performed by the Ljubljana group in the early 2000s (136, 137) demonstrated that the index of reactivation of the catalytic subunit of telomerase hTERT followed a pattern of progression that matched the LC grading system. Statistical analysis revealed significant differences at the level of AH as compared with BPCH. The group recently completed its studies of over 1,200 patients, covering a period of 25 years, proving that 9.5% of patients with AH progressed to SCC, whereas only 1.1% of the patients with SH/BPCH did, strongly justifying the predictive value of the LC (138).

From the diagnostic point of view, the key innovative contribution of the LC is the recognition of the histopathological features of BPCH and its clear-cut separation from atypical hyperplasia. The precise identification of BPCH is of utmost relevance in order to avoid over-diagnosis and potentially unnecessary treatment of patients with precursor lesions. The main problem when comparing dysplasia and squamous intraepithelial neoplasia (SIN) systems with LC grading is the lack of recognition of BPCH by the former two, as proposed by the WHO in 2005 (130). BPCH is a benign lesion that does not match either biologically or histologically with the concepts of mild dysplasia or SIN1 (130) because mild dysplasia and SIN1 represent lower grades of premalignant lesions. As early as 1995 the nuclear accumulation of p53 in low-grade dysplasia lesions was reported within a range similar to other frankly benign lesions; in contrast, high-grade dysplasia, which equates to atypical hyperplasia, presented a significantly higher p53 nuclear accumulation, which was very similar to that of SCC (13). At the stage of mild dysplasia, its mitochondrial DNA content increase, used as a measure of progression in HNSCC, showed a ratio similar to that of normal mucosa; it was minimally increased in moderate dysplasia and it was twice as high in severe dysplasia (139). Fortunately, good agreement exists on the most advanced stages of both grading systems; AH, or "risky epithelium" of the LC, bears an analogy to moderate and severe dysplasia, and CIS has similar significance in both systems.

In the HPV era, it is our view that the controversy of the last years between the advocates of the LC and those in favor of the dysplasia system will be soon a thing of the past. The LC will continue to prove its better predictive value for grading lesions developing

in heavy cigarette smokers and alcohol drinkers, as already postulated in 1999 by Hellquist et al. (134). At the same time, the dysplasia and SIN systems, whose criteria for grading head and neck precursors of HNSCC follow steps similar to those used by the WHO in 2003 for the uterine cervix (140), will maintain their indisputable value for grading intraepithelial precursor lesions related to the increasingly detected epidemic of HNSCC associated with high-risk HPV infections (7).

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