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Premalignant Lesions of the Upper Aerodigestive Tract: Biomarkers of Genetic Alterations, Proliferation, and Differentiation

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Abstract The normal distribution of cell division in squamous mucosa is in the basal or adjacent suprabasal cell layers. Migration of cells toward the epithelial surface results in cell differentiation, most often expressed by high molecular weight keratin intermediate filaments and components of the cornified envelope, including "involucrin." These latter expressions of terminal differentiation are common in keratinizing dysplasia and invasive squamous cell carcinomas. However, they are less common in the non-keratinizing dysplasias, which fail to express evidence of epithelial maturation. Cell proliferation occurs in or near the basal layer in normal or reactive/reversible hyperplasias. In dysplasia (both keratinizing and non-keratinizing), cell proliferation is observed at all levels of the epithelium. Concomitant with these abnormalities in proliferation and differentiation are nuclear changes characterized by large hyperchromatic nuclei. The enlarged nuclei reflect increased DNA content, as documented by flow cytometry and image analysis techniques. DNA aneuploidy represents a spectrum of genomic alterations reflecting steps toward the progression to invasive carcinoma, which for the most part, have not yet been identified.

Key words: Proliferation, differentiation, oncogene, growth factors, upper aerodigestive tract, biomarkers, premalignant

It is widely accepted that malignant and metastatic phenotypes are acquired in a progressive, stepwise fashion due to multiple, discrete genetic alterations. Numerous recent studies have elucidated some of the key *molecular level* events in neoplastic transformation and progression by evaluating abnormal structure and expression of single genes. As we have seen, the preinvasive stage of squamous intraepithelial neoplasia (SIN) is also reflected at the *histologic level* as a combination of deregulated proliferation, incomplete or inappropriate differentiation, and nuclear changes compatible with abnormal chromatin content or structure (*i.e.*, dysplasia). Moreover, we defined various histologic levels of severity (*i.e.*, predisposition to subsequent invasion), suggesting that accumulated genetic changes may have morphologic correlates. In addition to histologic and molecular level derangements, neoplastic progression may also be analyzed at the *cellular level*, by assessment or quantitation of DNA content, differentiation markers, and cell cycle distribution. These changes are represented by histologic alterations and will form the basis of discussion for this paper. Dissecting the cellular and molecular anomalies in SIN is difficult, owing to the limited availability of appropriately handled tissue, and the absolute requirement for histologic correlation, as well as obvious ethical considerations relating to serial sampling of human subjects.

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CYTOPHOTOMETRIC DNA ANALYSIS

Exact multiples of cellular DNA (*e.g.*, tetraploidy, polyploidy) is physiologic in some human cells (hepatocytes, transitional umbrella cells). However, partial genomic loss or duplication (aneuploidy) is always pathologic and represents a hallmark of neoplastic transformation. Cytogenetic analyses demonstrate that abnormalities of DNA structure and content in solid tumors are profound; however, traditional karyotypic analyses are cumbersome and often lack both specificity and sensitivity. In recent years, numerous investigators have approached cellular DNA content analysis using cytophotometric techniques. Cytophotometric DNA quantitation methods, including flow cytometry (FCM) and image analysis (IA), estimate relative levels of DNA-specific dyes which bind stoichiometrically. FCM performs rapid, automated fluorescence intensity measurements of disaggregated cells in suspension. Despite its speed and high level of precision, FCM is poorly adapted to evaluate focal lesions with limited cell numbers. DNA quantitation of preinvasive lesions, therefore, has most commonly been performed using IA, using manually-directed, optical density measurements made on Feulgen-stained nuclei.

The sensitivity of cytophotometric DNA analysis is approximately 5% of normal DNA content, or $\pm$1–2 chromosomes. Therefore, cytogenetic abnormalities such as small deletions and reciprocal translocations are not cytophotometrically detectable. Further, widespread karyotypic changes involving multiple chromosomes have been shown to result in diploid-range DNA content, especially if there is minimal net gain or loss of overall cellular DNA. For these reasons, DNA content analysis should not be over interpreted as a diagnostic procedure.

Abnormal DNA content correlates well with severity of dysplasia in upper aerodigestive tract (UADT) SIN lesions [1–4]. In our series, DNA aneuploidy was observed in 33% of mild dysplasias, 78% of moderate dysplasias, and 100% of severe dysplasias [5]. Others have reported similar associations between ploidy and histologic grade in dysplastic lesions of the cervix [6]. Interestingly, Hellquist *et al.* [7] also reported that keratinizing dysplasias often harbored DNA aneuploidy despite their "differentiated" appearance. These findings confirm the genetically unstable character of some histologically deceptive SIN lesions.

Invasive squamous carcinomas are not uniformly DNA aneuploid. In our recent survey of the literature [8], DNA aneuploidy was reported in 60–85% of UADT carcinomas. Genetic alterations resulting in abnormal DNA content are an early event in the clinical course of squamous neoplasia. In addition, it is likely that genetic changes are widespread within neoplastic populations prior to host invasion.

DNA content abnormalities in neoplastic tissues are generally *clonal*. In other words, the analysis identifies a discrete population of cells having similar but abnormal DNA content relative to normal control populations. These populations most often possess a DNA content between normal (diploid) and tetraploid cells. Some investigators have called attention to isolated events (*i.e.*, cells) with hypertetraploid DNA content. Bocking *et al.* [9] reported that these events become more common with increasing grades of dysplasia. These findings correlate with serial DNA measurements performed in the cervical mucosa of carcinogen-treated mice, in which increasing grades of dysplasia accompanied greater numbers of hypertetraploid events [10].

Apart from the implication that neoplastic progression may have cytophotometrically defined stages, "polyploidization" steps may represent one mechanism of aneuploid clone generation. Progression from polyploidy to aneuploidy may then occur through chromosome loss in subsequent mitotic divisions. Interestingly, Fu *et al.* [11] have correlated cytophotometric aneuploidy with the presence of morphologically abnormal mitotic figures in dysplastic lesions of the cervix, an observation which supports this notion. It is also true that DNA aneuploid populations generally have greater proliferation fractions than diploid-range clones. Frequent cell division, apart from being a consistent abnormality in dysplastic mucosa, may also represent a critical driving force in the genetic instability which characterizes neoplastic tissue (*i.e.*, via mitosis-associated mutations).

The previously noted clonal nature of cytophotometric DNA content anomalies suggests these technologies may be useful as a means to study tumor heterogeneity and clonal evolution. In general, studies of this type are underrepre-

sented in the cytometry literature because of the difficulties encountered with serial sampling and tissue microdissection. In one study of cervical intraepithelial neoplasia, Fu *et al.* [12] reported that microinvasive foci often displayed a lower DNA content relative to the surface dysplastic foci from which they were derived. Others compared *in situ* and invasive cervical neoplasia by flow cytometry, and found a high level of DNA content homogeneity [13]. There is little, if any, published work of this nature in the UADT SIN literature.

Although cytophotometric DNA content has been shown to be a prognostic factor in invasive UADT squamous carcinoma, there are no good studies correlating DNA analysis with progression or recurrence of SIN lesions. Published reports of cervical lesions, however, suggest analyses of this type may have clinical utility [6,14].

PROLIFERATION

In normal epithelium, proliferation and differentiation are integrally related [15]. Cellular differentiation beyond a certain point is associated with irreversible growth arrest. Conversely, cells with proliferative potential are unable to terminally differentiate. Cell cycling in normal mucosal tissue is also limited anatomically to the two cell layers immediately above the basal lamina, where pre-differentiated reserve cells are located. It should be noted that epithelial growth control is profoundly affected by chemical messages emanating from the extracellular matrix and stromal fibroblasts. Accordingly, growth factor receptors such as epidermal growth factor receptor (EGF-R) are expressed only by basal cells in normal epithelia.

Hyperplasia and dysplasia are similar to the extent that both are characterized by hyperproliferation. As noted in our paper reviewing UADT precursor lesion; [Crissman *et al.*, this volume] dysplasia is essentially defined as an uncoupling of proliferation and differentiation. Suprabasilar mitotic figures represent a cardinal histologic manifestation of SIN. Recent studies employing immunohistologic detection of cell cycle-specific proteins, such as proliferating cell nuclear antigen (PCNA), highlight conventional pathologic observations of altered proliferation in dysplasias. PCNA, also referred to as cyclin, is a DNA polymerase normally present in nuclei only during DNA replication prior to cell division (*i.e.*, synthesis phase). Dysplastic mucosal surfaces display PCNA-positive cells in large numbers and in more superficial cell layers as compared with normal. Not unexpectedly, the proportion of PCNA-positive cells correlates with grade of dysplasia [16,17]. Other methods of analyzing cell proliferation in mucosal biopsies are available. In addition to PCNA, Ki-67 and p102 are putative cell cycle-specific antigens. Commercially available antibodies directed against these antigens can quantitate growth fractions. A noteworthy *caveat* in all immunohistologic analyses of cell proliferation is the fixation-dependence of antigen expression [18], which mandates careful, uniform tissue handling.

Although studies of UADT SIN are limited, some interesting observations have been made about proliferation in other tissue epithelium. Several authors report that suprabasilar hyperproliferation may precede adenomatous changes in colorectal mucosa. Similar abnormal proliferation patterns have been observed in foci of squamous metaplasia adjacent to lung cancers [19]. These findings underscore the critical relevance of proliferation deregulation in epithelial malignancy, even at their earliest perceptible evolutionary stages.

Growth factors and proteins encoded by some oncogenes or tumor suppressor genes mediate or regulate rate-limiting steps in cell proliferation. EGF-R for example, is normally expressed on the plasma membrane of basal cells in squamous mucosa. This distribution is logical considering the stromal origin of (physiologic) growth factors and the strict limitation of cell proliferation to predifferentiated basal cells. EGF-R expression has been demonstrated in superficial cell layers of laryngeal SIN lesions [20]. The degree of aberrant EGF-R expression, moreover, correlates with increasing severity of dysplasia. This finding provides one explanation for the greater proliferative capacity of higher grade SIN, as previously noted. It has been proposed that constitutive growth factor receptor expression provides a means for autocrine growth stimulation in neoplastic populations, uncoupling them from the need for, or dependence on, environmental signals.

Mutational deactivation of p53, a proliferation-related tumor suppressor gene product, has

also been reported in UADT dysplasias. Wild-type p53 is a dimeric phosphoprotein believed to inhibit cell cycling via DNA binding interactions. A number of somatic inactivating point mutations have been described in many human solid tumor systems, including lung and esophageal carcinomas. Tumor suppressor loci, such as p53, may also be inactivated by chromosomal loss [21]. Coltrera *et al.* [16] recently correlated mutant p53 expression with increasing severity of grades of dysplasia in the UADT. Therefore, tumor suppressor gene inactivation may represent a second mechanism of deregulating cell cycle control. Presence of mutational tumor suppressor inactivation in preinvasive UADT neoplasia correlates with widespread genomic alterations implied by cytophotometric DNA analysis studies already cited. Indeed, the degree of molecular genetic changes (so-called allelic losses) has been correlated to aneuploidy in some tumor systems.

Abnormal expression of oncogenes with cell proliferation-related activities has been investigated in UADT squamous carcinoma, including c-*myc* and c-Ha-*ras* [22,23]. These studies have not been extended to SIN. Correlations between inappropriate growth factor, or tumor suppressor gene product expression, and advancing SIN lesions suggest such studies might be useful to identify dysplasias with a high risk of progression to invasive disease. To date, this has not been systematically investigated.

MARKERS OF SQUAMOUS DIFFERENTIATION

Apparent from our discussion of histologic precursors of squamous carcinoma [Crissman *et al.*, this volume] abnormal differentiation is a defining manifestation of UADT SIN. In normal squamous epithelia, profound cellular alterations accompany migration from basal layer to surface, reflecting sequential, orderly modifications of cell shape, cytoplasmic constituents, and plasma membrane. Dysplasias are predictably characterized by qualitative or quantitative aberrations of virtually all molecules involved in this exquisitely controlled process.

Basal lamina (BL) is an integral component of all epithelia. It serves to orient epithelial cells and anchor them to the underlying stroma. Under physiologic conditions, epithelial proliferation is limited to cells which contact the BL. BL is a macromolecular complex composed of type IV collagen, laminin, heparin sulfate, fibronectin, and proteoglycans. In tissue sections, it may be identified immunohistochemically by staining for the former components.

Normal epithelium has a continuous, uniform, well-defined BL, which is also well preserved in SIN I lesions. SIN III, in contrast, has thinned or absent BL, suggesting loss of BL maintenance or synthesis accompanied by increasing severity of SIN. These morphologic observations of clinical biopsies have been recently confirmed in a mouse skin carcinogenesis model [24]. Given the critical role of BL in physiologic growth and architecture of epithelial surfaces, loss of BL in SIN lesions should be regarded as evidence of profound functional abnormalities.

BL is often produced in squamous carcinomas after tumor cells invade adjacent submucosa and stromal tissue. It is usually found in well-differentiated foci, especially where there is evidence of "maturation" as expressed by keratinization. The most likely explanation is that active invasion occurs incrementally in most carcinomas, followed by architectural and functional changes simulating physiologic equilibrium [25]. Recently, we have also demonstrated an inverse relationship between cell proliferation (measured by PCNA/cyclin immunostaining) and BL staining in squamous carcinomas. These data confirm that relationships between proliferation and differentiation are perturbed, but not totally abrogated, in most squamous carcinomas. They also confirm the importance of BL as a relevant marker for differentiation [26].

There are numerous other markers of intracellular differentiation, including intermediate filament composition and keratinization-associated proteins. Normally, low molecular weight cytokeratin intermediate filaments are expressed in the basal cell compartment [18]. Conversion to higher molecular weight cytokeratins occurs as the cells "mature" into superficial layers, accounting for the characteristic orange, refractile staining in conventional tissue sections used in routine pathology. These high molecular weight keratins are components of the spinous and granular cell layers, the major protective components of squamous mucosa.

The cornified envelope, an impermeable subplasmalemmal complex, develops from involucrin, filaggrin, and other components which eventually bind with keratin complexes to form the *stratum corneum* [27].

A number of investigators have monitored these functional markers of squamous differentiation to detect early changes of intraepithelial neoplastic transformation, and have attempted to quantitate the degree of dysplastic change (28–31). Predictably, markers of differentiation are less pronounced, or abnormally distributed, in severe SIN. Conversely, markers of predifferentiated cells become more prominent. One recent study revealed suprabasal low molecular weight cytokeratin (CK19) in severe dysplasia/CIS (SIN III) biopsies [32]. Similar findings have been observed in the pyriform fossa [33] and cervix [34]. The functional relevance of intermediate filaments has not yet been elucidated. The role of cytokeratins in tumor progression, apart from a marker of predifferentiated cells, remains controversial.

Other "biomarkers" indicative of epithelial maturation include cell surface integrin receptors [24] and cell adhesion molecules such as E-cadherin [35]. These markers are expressed in normally differentiated epithelium, and mediate attachment to other epithelial cells or the extracellular matrix (*i.e.*, BL). Like other differentiation-associated cellular elements, they are either lost (not expressed) or expressed inappropriately in the basal-suprabasal layers. Abnormal cell adhesion is a critically important mechanism of both invasion and metastasis of solid tumors. Invading cells must not only detach from neighbors, but also establish contact with various extracellular matrix molecules. These functional characteristics are acquired in part through loss of normal intercellular connections. Ultrastructural morphometric studies have confirmed progressive loss of desmosomal cell junctions with increasing grades of uterine cervical dysplasia and with invasion in transitional carcinoma [36,37].

SUMMARY

Preinvasive neoplasia represents a deregulation in uncoupling proliferation and differentiation. The recent explosion of molecular genetic research has elucidated many, but certainly not all, of the individual genetic foci responsible for physiologic control of this process. In some adult tumor systems, detailed studies demonstrate convergence of genetic and histologic tumor progression. Theoretically, both genetic and histologic levels of pathology are ultimately reflected at the functional, cellular level. However, application of cellular pathology to clinically relevant issues in UADT SIN is at a preliminary stage. Technologies available for evaluation of proliferation and differentiation are incompletely developed and difficult to quantitate. Further, they have generally not been applied systematically in careful, follow-up series.

Refinements to the analysis of proliferation, chromosome aneuploidy, and cellular differentiation are rapidly becoming available, and will no doubt be applied to the appropriate clinical material. Neoplastic progression of adult solid tumor systems is complex, heterogeneous, and at least partially dependent on host factors. Therefore, it is unlikely that any *single* parameter, no matter how elegantly and precisely defined, will provide answers to difficult clinical problems in every patient. Malignant phenotypes, in contrast, most likely represent an interaction of numerous functional derangements, which do not necessarily arise in a consistent, prescribed sequence.

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