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ABSTRACT: The concept of a two-step process of cancer development in the oral mucosa, *i.e.*, the initial presence of a precursor subsequently developing into cancer, is well-established. Oral leukoplakia is the best-known precursor lesion. The evidence that oral leukoplakias are pre-malignant is mainly derived from follow-up studies showing that between < 1 and 18% of oral pre-malignant lesions will develop into oral cancer; it has been shown that certain clinical sub-types of leukoplakia are at a higher risk for malignant transformation than others. The presence of epithelial dysplasia may be even more important in predicting malignant development than the clinical characteristics. Three major problems, however, are attached to the importance of epithelial dysplasia in predicting malignant development: (1) The diagnosis is essentially subjective, (2) it seems that not all lesions exhibiting dysplasia will eventually become malignant and some may even regress, and (3) carcinoma can develop from lesions in which epithelial dysplasia was not diagnosed in previous biopsies. There is, therefore, a substantial need to improve the histologic assessment of epithelial dysplasia or, since epithelial dysplasia does not seem to be invariably associated with or even a necessary prerequisite for malignant development, it may be necessary to develop other methods for predicting the malignant potential of pre-malignant lesions. As a consequence of these problems, numerous attempts have been made to relate biological characteristics to the malignant potential of leukoplakias. Molecular biological markers have been suggested to be of value in the diagnosis and prognostic evaluation of leukoplakias. Markers of epithelial differentiation and, more recently, genomic markers could potentially be good candidates for improving the prognostic evaluation of precursors of oral cancer. As yet, one or a panel of molecular markers has not been determined that allows for a prognostic prediction of oral pre-cancer which is any more reliable than dysplasia recording. However, these new markers could be considered complementary to conventional prognostic evaluation.

Key words. Leukoplakia, oral pre-cancer, epithelial dysplasia, markers, prognosis.

(I) Introduction

The concept of a two-step process of cancer development in the oral mucosa, *i.e.*, the initial presence of a precursor (pre-malignant, pre-cancerous) lesion subsequently developing into cancer, is well-established. Oral leukoplakia is the best-known precursor lesion. It is not known how many oral squamous cell carcinomas arise from precursor lesions and how many develop from apparently normal oral mucosa. However, studies have shown that between 16 and 62% of oral carcinomas are associated with leukoplakic lesions when diagnosed (Bouquot *et al*., 1988; Gundlach, 1992; Scheifele and Reichart, 1998; Schepman *et al*., 1999), and an Indian house-to-house survey showed that about 80% of oral cancers were preceded by oral pre-cancerous lesions or conditions (Gupta *et al*., 1989). Others consider the vast majority of oral cancers to arise from otherwise clinically normal mucosa (*e.g.*, Cowan *et al*., 2001).

The evidence that oral leukoplakias are pre-malignant are mainly derived from follow-up studies, mostly obtained on hospital-based observations. Studies have shown that between < 1 and 18% of oral pre-malignant lesions will develop into oral cancer (Table 1). The transformation rates are generally lower in house-to-house surveys and in studies on random samples (Silverman *et al*., 1976; Gupta *et al*., 1980) than in studies on hospital-based patient populations, indicating the influence of case selection; the influence of geographical traits related to habits and genetics may play a role, since the population-based studies have been primarily performed in India. Thus, there is considerable uncertainty as to whether or not all clinically detectable lesions characterized as precursors will eventually develop into carcinoma.

When evaluating studies on the outcome of pre-malignant lesions after a follow-up period, including studies on the usability of molecular markers, one must recognize that the outcome can be influenced by treatment intervention which in turn can affect the reliability of the results obtained. In most centers, lesions exhibiting epithelial dysplasia, at least of moderate or severe grade, are excised, to diminish the risk for further malignant development. The same applies to lesions localized in presumed risk sites or in patients (heavy smokers and/or drinkers) at high risk for cancer development. From studies on the outcome of treatment of oral pre-malignant lesions by excision, it appears, however, that the risk of malignant development may not change significantly (McCartan, 1998; Schepman *et al*., 1998). Recurrences are seen in 10-20% and cancer development in 3-9% of areas of excised lesions (Vedtofte *et al*., 1987; Chiesa *et al*., 1993; Schoelch *et al*., 1999a). In a recent study, carcinoma development in treated and untreated leukoplakias was compared (Saito *et al*., 2001). Therapy was not randomized; most small lesions underwent surgical excision, whereas large lesions apparently were not surgically removed unless severely dysplastic. Among 75 surgically treated leukoplakias, one patient later developed a carcinoma, whereas among 51 leukoplakias that did not receive

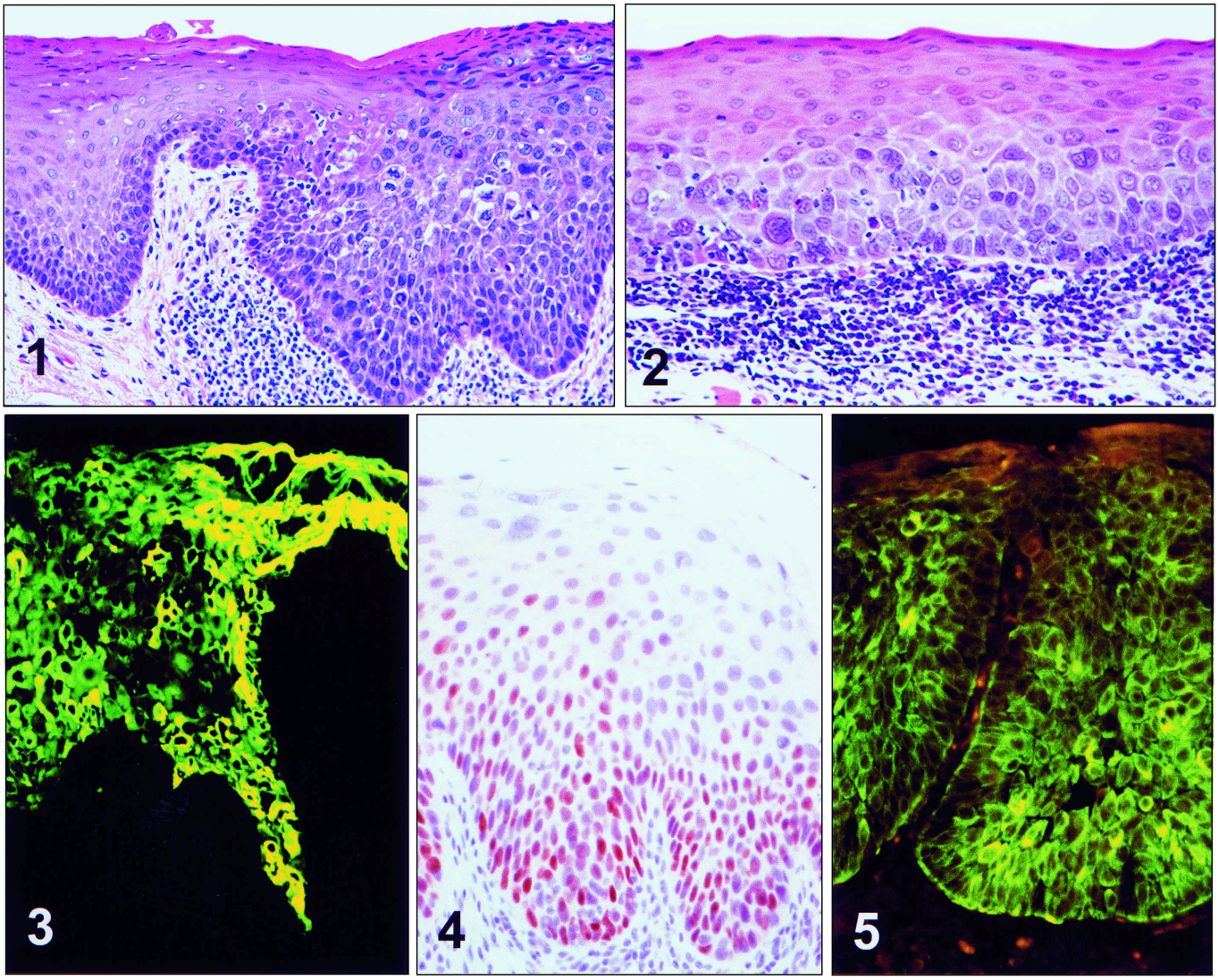

Figure 1. Biopsy of leukoplakia in floor of mouth showing severe dysplasia/carcinoma *in situ*. Note normal epithelium in left side. The dysplastic area is especially characterized by an increased nuclear-cytoplasmic ratio, an increased number of mitotic figures including abnormal mitoses and mitoses occurring in the middle and upper parts of the epithelium, nuclear hyperchromatism, and enlarged nuclei. H&E, X90.

Figure 2. Biopsy of leukoplakia at lateral border of the tongue showing mild to moderate epithelial dysplasia. Note normal stratification and cytology in superficial half of the epithelium. H&E, X190.

Figure 3. Pyogenic (teleangiectatic) granuloma on the gingiva stained for keratin 19. Note staining in almost the entire epithelium which represents pathologically non-cornified oral gingival epithelium. In epithelial dysplasia, a staining pattern similar to this one has been reported; however, in normal oral non-cornified epithelia, keratin 19 is variably detectable in the basal cell layer only. X100.

Figure 4. Biopsy of leukoplakia in floor of mouth stained for p53. Conventional histology showed moderate to severe dysplasia. Note staining in nuclei in basal and parabasal/spinous cells. X190.

Figure 5. Biopsy of leukoplakia in buccal mucosa stained for keratin 8. Conventional histology showed severe dysplasia/carcinoma *in situ*. Note staining in almost the entire thickness of the epithelium. In normal buccal epithelium, keratin 8 is not detectable by immunohistochemistry. X150.

treatment, four patients developed carcinoma. Thus, the carcinoma development in this material may be related to size of the lesion in addition to the lack of treatment. Previously, these same investigators (Saito *et al*., 1999) suggested that widespread leukoplakias had a higher rate of malignant transformation (Saito *et al*., 1999). Thus, although treatment undoubtedly influences the end-point in studies on the usability of markers, witholding treatment is not an option for obvious ethical reasons.

The term 'epithelial dysplasia' is assigned to histopathological changes associated with an increased risk of malignant development (*i.e.*, squamous cell carcinoma). The individual cellular changes are referred to as atypia, and the criteria customarily applied appear in Table 2 (Pindborg *et al*., 1997) (Figs. 1, 2).

Oral epithelial dysplasia is not associated with any specific clinical appearance. However, leukoplakia and erythroplakia are the lesions classically associated with dysplastic changes. Thus white, red, or mixed white and red changes are those most frequently revealing epithelial dysplasia. The frequency of epithelial dysplasia in leukoplakia varies between < 1 and > 30% (Mehta *et al*., 1969; Bánóczy and Csiba, 1976; Waldron and Shafer, 1975; Silverman *et al*., 1976, 1984; Burkhardt and Seifert, 1977; Eversole and Shopper, 1981; Lind, 1987). The lowest fre-

quency reported (Silverman *et al*., 1976) originated from a population-based study in India in which all clinically diagnosed leukoplakias were biopsied, emphasizing again the dependency on case selection and, possibly, geographical variations. Erythroplakia, a much more rare lesion than leukoplakia, almost invariably reveals epithelial dysplasia or frank carcinoma (Shafer and Waldron, 1975).

The presence of epithelial dysplasia is generally accepted as one of the most important predictors of malignant development in pre-malignant lesions. This was originally based on follow-up studies on cervical lesions (Richart and Barron, 1969), and later, on findings that oral lesions with epithelial dysplasia more often develop into carcinoma than those without dysplasia (Pindborg *et al*., 1977; Burkhardt and Maerker, 1978; Kramer *et al*., 1978; Gupta *et al*., 1980; Silverman *et al*., 1984; Lumerman *et al*., 1995; Lee *et al*., 2000; Cowan *et al*., 2001). Furthermore, features of epithelial dysplasia are commonly seen in epithelium adjacent to oral carcinomas (Katz *et al*., 1985). However, epithelial dysplasias will not necessarily develop into cancer, and some may even regress (Mincer *et al*., 1972; Silverman *et al*., 1976; Bánóczy, 1977; Pindborg *et al*., 1977; Gupta *et al*., 1980).

Thus, the challenge within the field of oral pre-cancer is to predict which lesions will eventually develop into carcinoma. Conventional clinical and histopathological aspects are not optimal for decisions on management, which is, of course, influenced by the perceived risk of malignant development. Resource-consuming management procedures might be spared if the risk of malignant development could be predicted with reasonable certainty and management of risk patients could be improved.

This article reviews the possibilities of predicting malignant development from precursor changes, in particular that represented by the clinical lesion diagnosed as leukoplakia and the histopathological change designated as epithelial dysplasia. Furthermore, in recent years our knowledge on the molecular biological characteristics of oral pre-cancer and cancer has increased dramatically and may, in the future, supplement clinical and histopathological parameters in evaluating prognosis.

(II) Terminology and Definitions

Terminology and definitions within the field of oral pre-cancer have been widely discussed. The use of the terms 'oral pre-cancer' and 'oral pre-malignancy' in itself poses problems, since this terminology signifies an invariable development of cancer from such diseases. The use of terms like 'potentially malignant' (Johnson *et al*., 1993) signifies more precisely what is actually meant. Notwithstanding the relevance of this discussion, the designations 'pre-cancer', 'pre-cancerous', 'pre-malignant', and 'precursors' will be used synonymously throughout this review for diseases with a malignant potential.

Several attempts to produce internationally accepted terminologies and definitions have appeared in the literature (WHO, 1978; Axéll *et al*., 1984, 1996; Pindborg *et al*., 1997; Meeting report, 1997).

TABLE 1
Selected Studies on Malignant Transformation of Oral Leukoplakia

Study/Year	Country	Material	Observation (yr)	% Malignant Transformation
Pindborg *et al.*, 1968	Denmark	248	3.9	4.4
Silverman and Rosen, 1968	USA	117	1-11	6.0
Kramer *et al.*, 1970	UK	187	?	4.8
Mehta *et al.*, 1972	India	117	10	0.9
Silverman *et al.*, 1976	India	4762	2	0.13
Bánóczy, 1977	Hungary	670	9.8	6.0
Silverman *et al.*, 1984	USA	257	7.2	17.5
Lind, 1987	Norway	157	9.3	8.9
Schepman *et al.*, 1998	Netherlands	166	2.5	12.0

The WHO (Pindborg *et al*., 1997) has accepted the latest international attempt on terminology and definitions (Axéll *et al*., 1996), subdividing oral pre-cancer into pre-cancerous lesions and pre-cancerous conditions. There is general agreement that a pre-cancerous lesion is defined as "a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart", whereas a pre-cancerous condition is defined as "a generalized state associated with a significantly increased risk of cancer". The latter definition signifies that the cancer can arise in any part of the oral cavity and not necessarily in a pre-existing lesion. Examples of pre-cancerous conditions are submucous fibrosis and lichen planus. The concept of field cancerization may weaken these definitions. A relatively high rate of second primaries is seen in the oral cavity (Winn and Blot, 1985; de Vries *et al*., 1986; Boysen *et al*., 1992); however, criteria are ill-defined. The concepts of second primaries and field cancerization are being re-evaluated based on new insights provided by molecular techniques, in particular genetic analysis (Califano *et al*., 1996; Partridge *et al*., 1997, 2000; Warnakulasuriya, 2002). Thus, a new classification has been proposed, encompassing second primary tumors, local recurrence, second field tumors (derived from the same

TABLE 2
Criteria Used for Diagnosing Epithelial Dysplasia (Pindborg *et al.*, 1997)

- Loss of polarity of basal cells
- The presence of more than one layer of cells having a basaloid appearance
- Increased nuclear-cytoplasmic ratio
- Drop-shaped rete ridges
- Irregular epithelial stratification
- Increased number of mitotic figures
- Mitotic figures that are abnormal in form
- The presence of mitotic figures in the superficial half of the epithelium
- Cellular and nuclear pleomorphism
- Nuclear hyperchromatism
- Enlarged nuclei
- Loss of intercellular adherence
- Keratinization of single cells or cell groups in the prickle cell layer

genetically altered field as the primary tumor), and metastases (Braakhuis *et al*., 2002).

Pre-cancerous lesions include leukoplakia and erythroplakia. Attempts at defining these lesions are presented in Table 3. There is general agreement that leukoplakia and erythroplakia are clinical diagnoses bearing no connotations as to their histopathology.

(III) Clinical Aspects Related to Malignant Potential

(A) Diagnosis

By excluding various lesions not believed to have a malignant potential from the conception of leukoplakia, we are left with a group of lesions with a higher malignant potential. The lesions to be excluded are lesions belonging to other entities, such as lichen planus (acknowledging that it has a malignant potential in itself), lupus erythematosus, leukoedema, and white sponge nevus, and lesions for which an etiology can be established, such as frictional keratosis, cheek/lip/tongue biting, contact lesions, and smoker's palate (Axéll *et al*., 1996; van der Waal *et al*., 1997). In many cases, a biopsy is mandatory to exclude such lesions. It is not always possible, however, to establish a suspected etiology with much certainty. If in doubt, one can make a provisional diagnosis of leukoplakia, and the definitive diagnosis can be established after the result of removal of any possible etiologic factors and a biopsy (Axéll *et al*., 1996; Pindborg *et al*., 1997; van der Waal *et al*., 1997).

Much of the variation in the assessments of the malignant transformation rates as illustrated in Table 1 are possibly related to the use of different inclusion criteria and, thereby, to the definition of oral leukoplakia used in individual studies (other possible causes are geographical variations [habits, genetic variations] and various follow-up periods). To make studies on oral leukoplakia more comparable, future studies should state in detail the inclusion criteria, including how the diagnoses were established (biopsy/exclusion of possible etiologic factors).

(B) Clinical appearance

Leukoplakias are divided into homogeneous and non-homogeneous ('speckled') types (Shafer and Waldron, 1961; Pindborg *et al*., 1963; Axéll *et al*., 1984) that can be further subdivided (Axéll *et al*., 1996). A very aggressive type of lesion, proliferative verrucous leukoplakia (Hansen *et al*., 1985), almost invariably develops into malignancy (Zakrzewska *et al*., 1996; Silverman and Gorsky, 1997). The clinical type of leukoplakia has a bearing on the prognosis, since the non-homogeneous leukoplakias containing an erythematous, nodular, and/or verrucous component have a higher malignant potential than the homogeneous ones (Pindborg *et al*., 1963, 1968; Bánóczy, 1977; Kramer *et al*., 1978; Silverman *et al*., 1984; Lind, 1987; Gupta *et al*., 1989; Schepman *et al*., 1998).

Although a four- to five-times-higher risk of malignant development is generally seen in the non-homogeneous leukoplakias compared with the homogeneous type, a malignant transformation rate of homogeneous leukoplakias of about 5% (Silverman *et al*., 1984; Lind, 1987) seems to warrant careful follow-up of these lesions as well (Kramer *et al*., 1978). As touched upon earlier, it should be noted that these transformation rates are based on selected patient populations. In a house-to-house survey in India (including solely tobacco users), only 0.6% of homogeneous leukoplakias developed into cancer (Gupta *et al*., 1989).

(C) Etiology

An established etiological factor for a given white or predominantly white lesion excludes a diagnosis of leukoplakia. Nevertheless, the use of tobacco and *Candida* infection are often mentioned as etiologic factors for leukoplakias, and both factors have been related to prognosis.

Smoking

The proportion of tobacco users among individuals with leukoplakia is high, and smoking cessation results in the disappearance of a substantial number of leukoplakias and in lower incidence rates for this lesion (Bánóczy, 1977; Baric *et al*., 1982; Roed-Petersen, 1982; Downer *et al*., 1995; Gupta *et al*., 1995; Bánóczy *et al*., 2001). According to the Axéll *et al*. (1984) definition of leukoplakia, an etiology related to tobacco use did not exclude a diagnosis of leukoplakia (Table 2). However, the most recent definition apparently does not include these lesions in the concept of leukoplakia (Axéll *et al*., 1996) (Table 2).

TABLE 3
International Attempts at Definition of Leukoplakia and Erythroplakia

	Leukoplakia	Erythroplakia
WHO, 1978	A white patch or plaque that cannot be characterized clinically or pathologically as any other disease	Bright red, velvety plaques which cannot be characterized clinically or pathologically as being due to any other condition.
Axéll *et al.*, 1984	A whitish patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except for the use of tobacco	Lesions of the oral mucosa that present as bright red patches or plaques that cannot be characterized clinically or pathologically as any other condition.
Axéll *et al.*, 1996	A predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion; some oral leukoplakias will transform into cancer.	Lesions of the oral mucosa that present as red areas and cannot be diagnosed as any other definable lesion.
Pindborg *et al.*, 1997 (WHO)	A predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion.	A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease.

Leukoplakias associated with a smoking habit seem to have less malignant potential than those not related to a smoking habit. In a study of 257 patients with oral leukoplakia, 183 were smokers of whom 12% developed carcinoma, whereas 74 were non-smokers of whom 32% developed carcinoma (Silverman *et al*., 1984). In another study of 166 patients with oral leukoplakia, women non-smokers had a significantly higher risk of malignant transformation than women smokers (Schepman *et al*., 1998). These results, as other similar findings, are remarkable, since smoking is the most important etiologic factor for carcinoma of the oral mucosa. It has been shown that there seems to be a strong relation between smoking and the development of leukoplakias in the floor of the mouth, whereas leukoplakias at the borders of the tongue are more common among non-smokers (Schepman *et al*., 2001). Since both of these sites are regarded as high-risk areas (see below), the relation between smoking and the risk of malignant development does not seem to depend on a difference in site predilection for smoking- and non-smoking-related leukoplakias.

If smoking can be established as an etiologic factor for a given white lesion, *i.e.*, the lesion disappears when the patient stops smoking, it has been argued that the lesion was not a leukoplakia at initial presentation. Since there are acknowledged difficulties in convincing patients to stop smoking, it would be of importance to be able to predict if a given white lesion would disappear upon smoking cessation. There is one characteristic clinical finding that indicates that a lesion is truly tobacco-induced: fine white striae that, taken together, imitate a fingerprint pattern in the mucosa (Pindborg *et al*., 1980). These lesions are referred to as 'fingerprint lesions' or a 'pumice stone' type of lesion. Histologically, these lesions show a typical chevron-like (also referred to as 'church-spire-' or 'Christmas tree-like') keratinization pattern. There is good evidence that these lesions will disappear upon tobacco cessation (Pindborg *et al*., 1980), and they are generally regarded as innocent lesions with no malignant potential. There is no evidence, however, that such fingerprint lesions could not develop into conventional types of leukoplakias, *i.e.*, lose their fingerprint pattern, and acquire a potential for malignant transformation. In an unknown number of cases, leukoplakias without a fingerprint pattern will also disappear upon smoking cessation. However, some lesions associated with a smoking habit but lacking the fingerprint pattern are possibly as dangerous as lesions not related to a smoking habit. It is possible that there is a continuum of changes which, at a certain point when the stem cells in the epithelium have been genetically damaged, become irreversible. Thus, the distinction between smoking-associated leukoplakias and 'idiopathic' leukoplakias in relation to prognosis in individual patients is questionable.

A variety of non-smoke tobacco habits has been reported in the literature, *e.g.*, snuff and different types of quids with tobacco products. A recent consensus report has proposed uniform reporting of quids and oral mucosal lesions resulting from some of these habits (Zain *et al*., 1999). The geographical variations in the habits reported and in the risk of malignancy associated with them are considerable. Snuff habits as they appear in Scandinavia seem to carry a very low risk of causing oral cancer (Axéll, 1993); however, some of the habits practiced in parts of Asia, Africa, and North America have been strongly related to cancer development (Mattson and Winn, 1989; Idris *et al*., 1996; X Zhang *et al*., 2001). These habits cause a range of white lesions which can be attributed to tobacco and/or other physical and chemical agents (Idris *et al*., 1996; Zain *et al*., 1999).

Candida infection

The association between *Candida* infection and the risk for malignant development originates from findings of an association between *Candida* infection and non-homogeneous leukoplakias (Jepsen and Winther, 1965; Renstrup, 1970; Bánóczy and Sugar, 1972) and lesions showing epithelial dysplasia (Renstrup, 1970; Hornstein and Grässel, 1992; Barrett *et al*., 1998). Moreover, a higher malignant transformation rate has been reported in *Candida*-infected leukoplakias (Roed-Petersen *et al*., 1970; Bánóczy and Sugar, 1972; Cawson and Binnie, 1980). Animal studies have indicated that *C. albicans* had an effect, in terms of the production of oral mucosal carcinomas in rats, similar to that of a known promoter in two-stage carcinogenesis experiments (O'Grady and Reade, 1992). Furthermore, the *Candida* types isolated from non-homogeneous leukoplakias seem to be of the more rare *C. albicans* types, some of which have a high nitrosation potential suggesting endogenous production of carcinogenic nitrosamines (Krogh *et al*., 1987a,b).

There is a longstanding discussion whether *Candida* infection is a cause of leukoplakia or if it is a superimposed infection in a pre-existing lesion (Jepsen and Winther, 1965; Cawson, 1966; Pindborg *et al*., 1968; Cawson and Binnie, 1980). It has been shown that, upon treatment, non-homogeneous *Candida*-infected leukoplakias convert into a homogeneous lesion, and some lesions even regressed (Holmstrup and Bessermann, 1983), which could be interpreted as support for both of the above suggestions. Experimental *Candida* infection in rats can produce whitish lesions with marked epithelial hyperplasia and epithelial atypia (Russell and Jones, 1975).

If one adheres to the internationally accepted definition of leukoplakia (Axéll *et al*., 1996; Pindborg *et al*., 1997), the discussion of an etiology for leukoplakias should be discouraged. The definition of leukoplakia rests on the symptom (clinical appearance) without any known etiology (definable disease). The wordings 'tobacco-induced leukoplakia' and '*Candida*-induced leukoplakia' are not consistent with the definition of leukoplakia. 'Smoking-associated leukoplakia' or '*Candida*-associated leukoplakia', in contrast, can be used to indicate a preliminary diagnosis of leukoplakia, while at the same time suggesting the implication of smoking or *Candida* infection as a possible etiologic agent in the development of the lesion. Diagnosis and treatment (including elimination of predisposing factors for *Candida* infection) of possible *Candida* infections in lesions suspected to be leukoplakias and/or smoking cessation should be instituted. The remaining lesion, if any, might then fulfill the criteria for a definite diagnosis of leukoplakia.

Other factors

The possible implication of human papilloma virus (HPV) in the etiology and potential for malignant transformation of oral pre-malignant lesions has been studied extensively (Nielsen *et al*., 1996; Praetorius, 1997; Miller and Johnstone, 2001). Results vary and are to a certain extent inconsistent. A recent meta-analysis (Miller and Johnstone, 2001) reported that the likelihood of detecting HPV was 2-3 times higher in pre-cancerous oral mucosa and 4-5 times higher in oral squamous cell carcinomas than in normal oral epithelium. This study also confirmed that high-risk HPVs (primarily type 16 or 18) were more

frequently associated with oral squamous cell carcinomas than low-risk HPVs. These findings must be interpreted in light of limitations, such as the problems in combining data from studies that are non-equivalent in terms of quality and methods (Miller and Johnstone, 2001). Further, such studies do not establish an etiologic role of HPV; however, there is sufficient evidence to justify further investigations with respect to this viral factor.

(D) LOCALIZATION

Studies have indicated that white lesions in the floor of the mouth and at the ventral surface of the tongue ('sublingual keratosis') have a higher malignant potential than leukoplakias in other regions of the oral mucosa (Kramer *et al*., 1978). In this latter study, 24% of 29 patients with sublingual keratosis affecting the floor of the mouth and/or the ventral surface of the tongue, followed for periods ranging from 1 to 19 yrs, developed carcinoma. A recent follow-up study of 166 leukoplakias (Schepman *et al*., 1998) has challenged this finding, since it was not possible to show a higher rate of malignant transformation of floor-of-the-mouth leukoplakias.

It is possible that the concept of high-risk sites may gain support from genetic studies. In a recent study, it was found that epithelial dysplasias from high-risk sites had a higher frequency of loss of heterozygosity and a pattern of loss associated with an increased risk of progression to malignancy (L Zhang *et al*., 2001). The study analyzed 71 epithelial dysplasias from the floor of the mouth, ventrolateral tongue, and soft palate, designated as high-risk sites, and 56 epithelial dysplasias from other sites of the oral cavity, designated as low-risk sites. The results were not influenced by gender or smoking.

Thus, the significance of the concept of high-risk sites comprising the floor of the mouth, ventrolateral tongue, and possibly the soft palate is unclear. Explanations for a possible existence of a higher risk in the floor of the mouth and ventrolateral tongue could be that these areas are substantially more exposed to carcinogens pooled in saliva than other areas of the oral cavity, and a that a higher permeability of the epithelium exists, as indicated in experimental studies on human oral mucosa (Lesch *et al*., 1989).

(IV) Histopathological Aspects Related to Malignant Potential—Epithelial Dysplasia

Epithelial dysplasia is defined as "a pre-cancerous lesion of stratified squamous epithelium characterized by cellular atypia and loss of normal maturation and stratification short of carcinoma *in situ*", whereas carcinoma *in situ* is defined as "a lesion in which the full thickness, or almost the full thickness, of squamous epithelium shows the cellular features of carcinoma without stromal invasion" (Pindborg *et al*., 1997). The criteria used for diagnosing these changes appear in Table 2, and "the more prominent or numerous they are, the more severe the grade of dysplasia" (Pindborg *et al*., 1997). On the basis of this rather loose statement, lesions are customarily graded into mild, moderate, and severe epithelial dysplasia.

Epithelial dysplasia can be found in biopsies of homogeneous leukoplakias, but is more commonly diagnosed in non-homogeneous leukoplakias (Shafer and Waldron, 1961; Pindborg *et al*., 1968; Mehta *et al*., 1969; Bánóczy and Csiba, 1976; Kaugars *et al*., 1988). Thus, there is an obvious congruence between the risk of malignant development from non-homogeneous leukoplakias and from lesions with epithelial dysplasia (Bánóczy and Csiba, 1976; Silverman *et al*., 1984). It is important to emphasize that although lesions with epithelial dysplasia more often develop into carcinoma than those without dysplasia, a large part of the carcinomas develop in lesions which did not show dysplasia in previous biopsies (*e.g*., Pindborg *et al*., 1977). Although wide inter-observer variations in scoring epithelial dysplasia have been reported, a recent study of 45 oral white patches without epithelial dysplasia supports this view, since 11% developed into cancer within a minimum follow-up time of 63 months (Sudbø *et al*., 2001b).

The significance of the subdivision of epithelial dysplasia has not been fully clarified. It has been reported that mild, moderate, and severe dysplasias develop into malignancy in 3%, 4%, and 43%, respectively (Burkhardt and Maerker, 1978). Some authors do not attach much significance to mild dysplastic changes. Thus, in a study on clinical characteristics of oral lesions showing epithelial dysplasia, the lowest degree of dysplasia was omitted from the experimental group (Mincer *et al*., 1972), and in other studies mild epithelial dysplasia is grouped with lesions showing no dysplasia (Cruz *et al*., 1998; Lee *et al*., 2000). The first of the latter two studies did not find any significant differences between the rate of malignant development from the group with no or mild dysplasia and the group with moderate and severe dysplasia, whereas the other study did. Another study combined mild and moderate dysplasia and severe dysplasia and carcinoma *in situ*, since the authors believed that these groupings carried the same prognostic significance (Waldron and Shafer, 1975). Thus, it is difficult to evaluate the significance of the different grades of epithelial dysplasia from the available literature, due to differing opinions on the subject and a lack of testing in well-defined multivariate analyses. A recent study of 150 patients with oral leukoplakia and another of 37 patients with oral erythroplakia histologically typed as dysplasia convincingly showed that histological grading into mild, moderate, and severe epithelial dysplasia had no significant value in predicting malignant development (Sudbø *et al*., 2001a, 2002).

For the pathologist, epithelial dysplasias/carcinomas *in situ* are probably more or less subconsciously defined as "histological changes in which the risk for development of a carcinoma is higher than in non-dysplastic epithelium". Several studies have shown great inter- and intra-examiner variability in the assessment of the presence or absence and the grade of oral epithelial dysplasia (Pindborg *et al*., 1985; Abbey *et al*., 1995; Karabulut *et al*., 1995; Sudbø *et al*., 2001a), the kappa values in general showing poor to moderate agreement among examiners. Although most oral pathologists possibly recognize and accept the criteria presented in Table 2, there is great variability in their interpretation of the presence, degree, and significance of the individual criteria.

Thus, from the above discussion, it appears that three major problems are attached to the importance of epithelial dysplasia in predicting malignant development: (1) Although a set of criteria for assessing epithelial dysplasia has been known for more than 20 years, the final diagnosis is, as discussed above, essentially subjective; (2) although an increased risk of malignant transformation in lesions showing epithelial dysplasia has been documented, it seems that not all of those lesions showing dysplasia will eventually become malignant, and some may even regress; and (3) carcinoma can develop from

lesions in which epithelial dysplasia has not been diagnosed in previous biopsies (Pindborg *et al*., 1977; Silverman *et al*., 1984; Schepman *et al*., 1998). These problems were recognized by the *WHO Collaborating Centre for Oral Pre-cancerous Lesions*, established in Copenhagen in 1967, and were emphasized in the report from the Centre published in 1978 (WHO, 1978).

Therefore, there is a substantial need to improve the histologic assessment of epithelial dysplasia, or, since epithelial dysplasia does not seem to be invariably associated with or even a necessary prerequisite for malignant development, it may be necessary to develop other methods for predicting the malignant potential of pre-malignant lesions.

(a) Is it possible to improve the histologic assessment of epithelial dysplasia?

The problems in evaluating the significance of epithelial dysplasia arise from a lack of objectivity in the evaluation of established criteria, arbitrary division of the gradings, lack of calibration of criteria and grading, and lack of sufficient knowledge of which criteria are important for the prediction of malignant potential (Warnakulasuriya, 2001). These difficulties have been known for decades and are also encountered in the scoring of dysplasia at other sites, such as the uterine cervix (Ringsted *et al*., 1978).

Several attempts have been made at objective assessment of the histologic presence or absence and of the grade of epithelial dysplasia. A scoring system based on a set of photographic standards was suggested in the late 1960s (Smith and Pindborg, 1969). The system was found to be of considerable value for purposes of standardization (Katz *et al*., 1985); however, the system relies on the weighting of the individual criteria originally made by the authors and, therefore, does not solve the problem of subjectivity and the need for identification of criteria known to be important for the prediction of malignant transformation. The system is rather laborious and has not gained wide use for routine diagnostic purposes. In some studies, criteria have been suggested for the subdivision of epithelial dysplasia into mild, moderate, and severe grades, based on the number of individual histologic features necessary to be present for diagnosis of the three grades or by an assessment of the extension of the cytological changes from the basal cell layer and upward (Bánóczy and Csiba, 1976; Krutchkoff *et al*., 1991; Lumerman *et al*., 1995; Speight *et al*., 1996). However, the significance of these attempts has not been studied, and difficulties in the use of either method are encountered, *e.g.*, the number and severity of histologic features necessary to be present for each grade and the problems related to epithelial thickness, transverse sectioning, and the undulating epithelial-connective interface in most regions of the oral mucosa. Reducing the number of grades from three to two might lead to more objectivity; however, the inter-examiner agreement did not increase when this was attempted (Sudbø *et al*., 2001a).

In some studies on examiner variability, knowledge of clinical characteristics was deliberately omitted (Abbey *et al*., 1995; Karabulut *et al*., 1995), whereas in another, an attempt was made to determine if knowledge of the clinical aspects would increase agreement rates (Abbey *et al*., 1998). This was not the case, however. Calibration was indirectly tested in one study (Karabulut *et al*., 1995), where two of four examiners had daily diagnostic discussions and agreements on the grade of dysplasia before signing off on a diagnosis. However, the two 'calibrated' examiners did not agree better with each other than with two other examiners. Furthermore, this study concluded that educational background (two oral pathologists with a dental background *vs.* two general pathologists with a medical background) did not explain the poor agreement rates. Studies from other organ systems have shown that inter-examiner agreements were no better between examiners with a specialist interest in the organ system tested than between other pairs of examiners (Eaden *et al*., 2001). Thus, it seems that the lack of objectivity in the evaluation of established criteria, *i.e.*, the arbitrary division of the gradings, and lack of effect of calibration or expertise are true obstacles that are difficult to deal with.

The importance of individual criteria in predicting malignant transformation has been dealt with by studies of epithelial dysplasia adjacent to oral carcinomas, based on an assumption that dysplastic features in this region were truly pre-malignant (Wright and Shear, 1985). The highest frequencies of dysplastic features found were basal cell hyperplasia, perturbation in epithelial maturation, and nuclear hyperchromatism. A recent study (Weijers *et al*., 2002) suggested that the presence of mild to moderate epithelial dysplasia in the margins of surgically removed oral squamous cell carcinomas carries a significant risk for local recurrence. Also, in support of the idea of examining dysplastic features adjacent to carcinomas are studies on the presence and distribution of markers such as keratins, p53, epidermal growth factor receptor, and chromosome instability that might be related to the carcinomatous process and the concept of field cancerization (Ogden *et al*., 1993; Brennan *et al*., 1995; van Oijen *et al*., 1998; Cruz *et al*., 2000; van der Toorn *et al*., 2001). One study, however, did not find the presence of p53 in the wound margin following excision of oral cancer predictive of second malignant tumors (Ogden *et al*., 1997).

By the use of discriminant analysis, criteria important for future malignant development have been studied (Kramer *et al*., 1970). Several of the classic criteria for epithelial dysplasia were found to be important; the three criteria that were given the heaviest weighting were abnormal mitotic figures in spinous and basal layers and disturbed polarity of epithelial cells. Surprisingly, Russell bodies in the lamina propria were also relatively heavily weighted. The findings of Kramer *et al*. (1970) with discriminant analysis have not been tested in subsequent large-scale studies for their malignant transformation predictability. Interestingly, similar methods of analysis are used today for monitoring the expression of thousands of genes during tumor progression by array technologies which may form a basis for a detailed molecular characterization of cancer development (Patel *et al*., 2001; Thykjaer *et al*., 2001).

It is of interest that reactive hyperplasia (denture-induced hyperplasia), a chronic inflammatory lesion (lichen planus), and benign tumors (squamous cell papillomas) may reveal mild degrees of dysplastic features when evaluated by the Smith-Pindborg system (MacDonald and Rennie, 1975), indicating that such changes may be reactive in nature. The features which were least evident were individual cell keratinization, bizzare mitoses, and mitoses at an abnormally superficial level in the epithelium, the first two of which were among features most important in distinguishing leukoplakias undergoing malignant development from other leukoplakias by the abovementioned discriminant analysis (Kramer *et al*., 1970). The abnormally situated mitoses, however, did not appear to be an important factor in the discriminant analysis.

It seems fair to conclude that epithelial dysplasia is an important marker of malignant development from pre-malig-

nant lesions; however, far from all dysplastic lesions will eventually develop into malignancy, and some may even regress or disappear. A set of criteria for diagnosing epithelial dysplasia has been known for more than 20 years, and it has been repeatedly shown that the evaluation and grading of epithelial dysplasia are very subjective, with poor to moderate agreement rates.

(V) Molecular Biological Aspects Related to Malignant Potential

A diagnosis of epithelial dysplasia is based on a static snapshot. In spite of this, this histologic diagnosis implies the possibility of a dynamic process, *i.e.*, subsequent malignant transformation. A better understanding of the fundamental molecular biology of the process of cancer development in the oral cavity through stages, conventionally defined as epithelial dysplasia/carcinoma *in situ*, may be the only way to improve our possibilities for predicting malignant development from precursor lesions. There are numerous reports on the application of molecular biological markers for the assessment of cancer risk, and recent reviews are available (Dabelsteen *et al*., 1991a,b; Johnson *et al*., 1993; Scully and Burkhardt, 1993; Warnakulasuriya and Johnson, 1996; Scully and Field, 1997; Moll and Schramm, 1998; Warnakulasuriya, 1998, 2000; Nylander *et al*., 2000; Park *et al*., 2000; Schwartz, 2000; Zhang and Rosin, 2001; Dabelsteen, 2002). When evaluating molecular changes in oral pre-malignancy and oral cancer, one should note that there are differences in the ethnic and etiologic characteristics in different parts of the world (Paterson *et al*., 1996).

Two approaches have been used for the study of markers of malignant development. In some studies, epithelial dysplasias on the one hand and oral cancers on the other are characterized with respect to the presence/absence or the pattern of distribution of the marker in question, and generally, the marker is characterized as a promising tool if the reaction pattern in epithelial dysplasias is similar to that in carcinomas and/or if the aberrant reaction pattern is positively related to the grade of epithelial dysplasia. In light of the subjectivity that exists in the diagnosis and grading of epithelial dysplasia, this approach is probably useful as a preliminary approach in the planning of further studies. Studies of markers thought to be directly related to malignant development, such as the expression of oncogenes or the loss of tumor suppressor genes, however, are of interest, although the outcome of the lesions examined is unknown. Other studies, mostly retrospective, compare the reaction pattern in pre-malignant lesions with the outcome (cancer or non-cancer) after a follow-up period. Despite the problems related to the retrospective nature of such studies and problems in gathering a substantial number of pre-malignant lesions, such studies should be encouraged.

Reference has earlier been made to the fact that reactive lesions and benign tumors sometimes display features of epithelial dysplasia (MacDonald and Rennie, 1975). The reaction patterns of molecular markers in such lesions are largely unknown; however, a few studies have shown that benign lesions in some cases reveal a reaction pattern similar to that seen in epithelial dysplasias and cancer. One study showed that hyperplastic oral epithelium in inflamed specimens (inflammatory papillary hyperplasia of the palate), which are rarely if ever associated with malignant development, exhibited a significant increase in positively stained cells for p53 and a proliferation marker (proliferating cell nuclear antigen [PCNA]) compared with normal palatal epithelium (Kaplan *et al*., 1998), and another study revealed a similar increase in cells stained positive for markers of cell cycle regulation (PCNA, Ki-67, AgNORs) in inflamed compared with non-inflamed keratocysts (de Paula *et al*., 2000). Furthermore, it has been shown that inflamed gingival epithelia exhibits a staining pattern for keratin 19 (Bosch *et al*., 1989) that has been described as characteristic of dysplastic epithelium (Lindberg and Rheinwald, 1989) (Fig. 3). Similar staining patterns for low-molecular-weight keratins have been reported in the reticular epithelium of the palatine tonsils in which a close relationship exists between epithelium and immunocompetent cells (Reibel and Sørensen, 1991). A recent study concluded that the pattern of keratin gene expression may be altered in response to frictional/smoking stimuli or immune-mediated mechanisms (Bloor *et al*., 2000). Expression of keratins 8 and 18 was reported to be amplified in gingival epithelia in the presence of inflammation (Pritlove-Carson *et al*., 1997), and discontinuities or disruptions in the staining patterns for type IV collagen and laminin in the basement membrane similar to those seen in epithelial dysplasia and cancer (Thorup *et al*., 1998) have been reported (Le Bars *et al*., 2001). The aforementioned changes have been shown to parallel the progression of oral epithelial neoplasia (Tosios *et al*., 1998). Thus, it is important to evaluate descriptive studies with caution; they do not necessarily reflect the biological significance of these molecular markers (Pyke *et al*., 1995).

Therefore, studies on molecular markers in epithelial dysplasias should include controls such as the normal counterparts of the dysplastic tissue, and inflamed tissues in which the inflammatory process seems to induce proliferative and differentiation-related changes mimicking those seen in pre-malignancy. The use of antigen retrieval methods that sometimes bring out false-positive reactions will possibly enhance the need for appropriate controls (Dowell and Ogden, 1996).

The best-characterized markers for determining future cancer development in oral pre-malignant lesions can be divided into: (1) genomic markers, including DNA content (ploidy), chromosome aberrations (allelic loss or gain), and changes in the expression of oncogenes and tumor suppressor genes (p53); (2) proliferation markers; and (3) differentiation markers, including keratins and carbohydrate antigens. The rationale for studying these markers in cancer development seems obvious. It is not within the scope of this review to describe the biological aspects of cancer development in any detail. However, examples of markers (genomic and differentiation) with a potential for predicting malignant transformation in oral pre-malignant lesions will be discussed. Admittedly, this field of research is rapidly evolving, and new potential predictive markers are probably on the horizon.

(A) DNA ANEUPLOIDY

The DNA content (DNA ploidy) of a cell gives a rough measurement of genetic instability and DNA aberration. In cancers, genetically stable diploid cells are replaced by genetically unstable aneuploid cells. In oral squamous cell carcinomas, DNA aneuploidy has been studied by flow and image cytometry, and the findings reveal that aneuploid tumor populations exist in a high number of cases and that the ploidy status is an important prognostic factor (Schimming *et al*., 1998). Other studies, however, did not find DNA ploidy status to have any prognostic value (Bundgaard *et al*., 1992). In oral leukoplakias, aneuploid populations have also been reported with (Steinbeck

et al., 1993; Saito *et al*., 1995) or without (Kahn *et al*., 1993; Högmo *et al*., 1996) correlation to the grade of dysplasia.

A recent impressive series of studies has focused on DNA ploidy measurements in patients with oral epithelial dysplasias during a rather long follow-up period (Sudbø *et al*., 2001a,b,c, 2002). DNA aneuploidy was a powerful predictor of malignant development in oral leukoplakias and erythroplakias, whereas normal DNA content indicated a low risk. The results are indeed promising; however, although the sample analyzed was comparatively large, the clinical value of this marker must be evaluated in large-scale prospective trials. Furthermore, there is a need for the development of simple methods for DNA measurements for routine diagnostic work.

(B) Loss of heterozygosity

Loss of genomic material in one of a pair of chromosomes is designated loss of heterozygosity (LOH). LOH at chromosomal regions supposed to contain tumor suppressor genes might be related to the process of malignant development, although it is recognized that the development of malignancies, in general, requires multiple genetic alterations (Renan, 1993). LOH in oral pre-malignant lesions and its possible predictive value were recently reviewed (Zhang and Rosin, 2001). LOH, in particular at chromosome arms 3p and 9p, was shown to be associated with a greater possibility of malignant development of pre-malignant lesions (Califano *et al*., 1996; Mao *et al*., 1996; Lee *et al*., 2000; Partridge *et al*., 2000; Rosin *et al*., 2000), although with longer follow-up the association weakened somewhat (Lee *et al*., 2000). Other chromosomal losses in addition to 3p and 9p increased the possibility of malignant development (Rosin *et al*., 2000). Thus, those lesions with LOH limited to 3p and/or 9p had a 3.8-fold increased risk, whereas those with loss at any of the chromosomes 4q, 8p, 11q, 13q, and 17p in addition to LOH at 3p and/or 9p had a 33-fold increased risk for progression to cancer compared with lesions that retained these arms. It has been shown, in the aerodigestive tract, that LOH at an increasing number of loci correlates with histopathological progression from benign squamous hyperplasia *via* dysplasia and carcinoma *in situ* to invasive carcinoma (Califano *et al*., 1996).

An increase in LOH in oral leukoplakia with foci of early cancerization (foci with superficial invasive growth) has been reported in these foci (Jiang *et al*., 2001). Furthermore, it appeared that, apart from the additional LOH, the chromosome arms lost in 11 of 13 cases in the non-invasive parts of the leukoplakias were also lost in the foci of early cancerization, thereby suggesting the concept of a single clone in the leukoplakia and invasive areas.

Reference was made earlier to a study on LOH in high-risk sites of oral leukoplakia (L Zhang *et al*., 2001) in which dysplasias at high-risk sites harbored significantly higher LOH frequencies than those at low-risk sites. This finding was applied in a comparison of lesions with mild and moderate epithelial dysplasia in high- and low-risk areas, respectively. However, there were no differences when severe dysplasias/carcinomas *in situ* at the two types of sites were compared. This suggests that when a stage of severe dysplasia/carcinoma *in situ* has been reached, the genetic disturbances are of such magnitude that they mask the influences of other possible risk factors.

LOH analysis has recently been suggested to be of value in differentiating verrucous hyperplasia/verrucous carcinoma from reactive lesions (Poh *et al*., 2001).

(C) p53

Mutation of the p53 tumor suppressor gene may represent the most common genetic change in human cancer (Greenblatt *et al*., 1994). The physiologic function of the p53 protein is that of preventing accumulation of genetic damage in cells either by allowing for repair of the damage before cell division or by causing death of the cell. The normal p53 protein has a very short half-life; the quantity in normal cells is extremely small. Therefore, it is usually not detectable by immunohistochemistry. Mutant p53 protein has a prolonged half-life and can accumulate in cells to levels that are detectable (Fig. 4). This mutant protein is normally not active, thus leading to the loss of the tumor suppressor function of the protein. More than 50% of oral squamous cell carcinomas are positive for p53 protein, and mutations of the p53 gene have been documented (for review, see Nylander *et al*., 2000). Furthermore, in oral epithelial dysplasias adjacent to oral carcinomas and in epithelial dysplasias not associated with oral carcinomas, overexpression of p53 protein and gene mutations have been detected as well (Warnakulasuriya and Johnson, 1992; Slootweg *et al*., 1994; Piffko *et al*., 1995; Califano *et al*., 1996). Several studies have compared p53 staining in pre-malignant lesions with subsequent malignant development (Regezi *et al*., 1995; Kushner *et al*., 1997; Rowley *et al*., 1997; Girod *et al*., 1998; Murti *et al*., 1998; Schoelch *et al*., 1999b,c; Shahnavaz *et al*., 2000). Various results were obtained, none of which points toward an established relationship between p53 overexpression in pre-malignant lesions and subsequent malignant development. Small samples make firm conclusions difficult, and a recent meta-analysis of published results from seven studies showed that 47% of oral pre-cancers had p53 overexpression (Warnakulasuriya, 1998)—a much higher percentage than the percentage of malignant transformation of pre-malignant lesions.

Interestingly, a recent study, taking into account the expression pattern of p53 within the epithelium, suggested that clear expression of p53 above the basal cell layer is an indicator of a developing carcinoma, even in the absence of obvious dysplasia (Cruz *et al*., 1998). However, it was strongly recommended that conventional histological parameters should also be taken into account, since p53 positivity is not always seen in lesions that undergo malignant transformation. In yet another study (Lee *et al*., 2000), parabasal p53-positivity was also associated with a higher cancer risk, even more so when combined with two other markers (chromosomal polysomy and loss of heterozygosity at chromosome 3p or 9p). This study, however, was performed on 'advanced' pre-malignant lesions and included patients with a previous oral cancer history.

At present, it is not possible to detect p53 protein selectively by immunohistochemistry. Thus, what is detected by immunohistochemistry may not be p53 gene mutation (Ranasinghe *et al*., 1993; Shahnavaz *et al*., 2000), but rather the stabilized normal protein. In other words, the correlation between cells that are positive for p53 and mutations of the genes in the same cells is still controversial. Immunohistochemical detection of p53 in pre-malignant lesions, therefore, is unlikely to be a reliable predictor—at least when used as a single marker (Warnakulasuriya, 2000).

A recent immunohistochemical study of p53 and p21, one of the downstream target genes activated by p53, in 53 oral verrucous leukoplakias reported that aberrant immunoreactivity of p53 and p21 was closely associated with malignant transfor-

mation (Chang *et al*., 2000). It has been reported earlier that, in most oral squamous cell carcinomas, p21 expression does not depend on p53 status (Warnakulasuriya *et al*., 1998), whereas in another study, the p21 expression seemed to correlate with p53 status (Piffko *et al*., 1999).

(D) Differentiation markers

Cell-surface carbohydrates

Cell-surface carbohydrates with blood group antigen activity are widely distributed in human tissues (Ravn and Dabelsteen, 2000). The term 'histo-blood group antigens' has been suggested for blood group antigens located on cells other than erythrocytes (Clausen and Hakomori, 1989). Histo-blood group antigens of the ABH, Lewis, and T/Tn systems are seen at the surfaces of epithelial cells in oral squamous epithelium (Dabelsteen *et al*., 1982, 1991b; Ravn and Dabelsteen, 2000). During cellular differentiation in stratified squamous epithelium, there is a sequential elongation of the terminal carbohydrate chain of precursors of histo-blood group antigens by the action of gene-encoded glycosyltransferases (Mandel *et al*., 1992).

During malignant development, the synthesis of histo-blood group antigens is disturbed (Dabelsteen *et al*., 1991a), possibly due to aberrant expression of the glycosyltransferases (Mandel *et al*., 1992, 1999). Almost 30 years ago, Dabelsteen and Pindborg (1973) showed that histo-blood group antigen A was lost in oral carcinomas. Further, in oral epithelial dysplasias, there was a loss of the normally expressed histo-blood group antigens (A or B) in the spinous cell layer, and an increased number of epithelial cell layers stained for the precursor molecule (H-antigen), which is normally expressed only in the parabasal cells (Dabelsteen *et al*., 1975, 1983). In normal epithelium, histo-blood group antigen Le^y is present on parabasal cells, whereas in epithelial dysplasias the expression of Le^y is seen in cell surfaces of the superficial spinous cells, possibly reflecting a lack of normal epithelial differentiation (Dabelsteen *et al*., 1988). A similar pattern of expression of simple mucin type carbohydrate antigens (T/Tn) has been reported in oral leukoplakias and erythroplakias (Bryne *et al*., 1991). Interestingly, mice genetically deficient in Muc2, a gastrointestinal mucin with a glycosylation pattern related to the T/Tn antigens, developed adenomas in the small intestine that progressed to adenocarcinomas, suggesting a role for this mucin in the suppression of cancer development (Velcich *et al*., 2002). Changes in histo-blood group antigen expression, similar to those in oral epithelial dysplasia, have been demonstrated in cancer development in the bladder (Orntoft, 1990).

Some of the aberrant expression patterns referred to above were seen in pre-malignant lesions without epithelial dysplasia (Dabelsteen *et al*., 1975; Bryne *et al*., 1991), suggesting that histo-blood group antigen changes appear early in the development of malignancy. However, only in a very limited number of cases have the histo-blood group antigen changes been related directly to the ultimate fate of the lesions (cancer/non-cancer) (Dabelsteen and Fulling, 1971; Dabelsteen *et al*., 1975). These studies showed that pre-malignant lesions that later developed into cancer exhibited a loss of histo-blood group antigen A years before malignant transformation. Preliminary studies indicate that this loss is due to allelic loss of the ABO glycosyltransferase-encoding genes, although post-transcriptional down-regulation of the gene transcript may also be involved (Gao *et al*., 2002). Some of the changes seen in pre-malignant and malignant lesions are also seen in non-malignant circumstances such as wound healing (Dabelsteen *et al*., 1998). Thus, the prognostic value of aberrant histo-blood group antigen expression in oral pre-malignant lesions is largely unknown. It should be mentioned, however, that, in experimental carcinogenesis in rat oral mucosa, changes in cell-surface carbohydrates were always seen in non-invasive lesions (Prime *et al*., 1987; Reibel *et al*., 1988). This is of interest, because all rats in this model were known to develop cancer if they were not killed. Furthermore, in this rat model, lesions classified as questionable epithelial dysplasia also revealed marked changes in the expression of histo-blood group antigens (Reibel *et al*., 1988). The histo-blood group antigen expression in this model paralleled morphological changes in malignant development. Furthermore, in cervical (To *et al*., 1986), head and neck (Carey *et al*., 1993), and oral carcinomas (Bryne *et al*., 1991), the expression of histo-blood group antigens has been shown to be related to prognosis. Several studies have shown that loss of A or B histo-blood group antigen expression is associated with increased motility of tumor cells, invasion in matrigel, and tumorigenicity in syngeneic animals (for review, see Dabelsteen, 2002). Thus, the prognostic value of the histo-blood group antigens as markers of malignant development in oral pre-malignant lesions in well-controlled follow-up studies is warranted.

Keratins

Keratins are proteins that constitute the intermediate filament cytoskeleton of epithelial cells. About 20 keratins are known, and they have been numbered 1-20. In the oral squamous epithelium, a certain set of keratins is present under normal circumstances; however, during malignant development, changes in the type or distribution of keratins are seen. In all normal oral epithelia, K5/K14 is present in the basal cell layer, whereas K4/K13 and K1/K10 are present in the spinous cell layer in non-cornified and cornified epithelium, respectively (Morgan *et al*., 1987). In general, the distribution of keratin mRNAs involves a higher number of epithelial cell layers than the corresponding proteins, indicating that these genes are under post-transcriptional control (Bloor *et al*., 2001).

The K5/K14 keratins that normally are present only in the basal cell layer are also expressed in the parabasal and spinous cell layers in dysplastic epithelia (Reibel *et al*., 1985; Vigneswaran *et al*., 1989; Heyden *et al*., 1992), probably reflecting the basal cell hyperplasia that is frequently seen in dysplasias. Furthermore, the keratins (K4/K13 or K1/K10) characteristically present in suprabasal cell layers show reduced expression or loss in epithelial dysplasias (Vigneswaran *et al*., 1989; Heyden *et al*., 1992; Su *et al*., 1996; Bloor *et al*., 2001). In one study (Bloor *et al*., 2001), a relation between the severity of dysplastic changes and altered keratin expression was demonstrated. Thus, in severe dysplasia, keratins (K4/K13 and K1/K10) associated with normal epithelial differentiation were almost completely lost.

In normal oral epithelium, keratins 8 and 18, normally expressed in simple epithelia, are generally not detected by immunohistochemistry, although their mRNAs are present in basal and lower spinous cells (Su *et al*., 1994). However, in oral epithelial dysplasias, these keratins were detected by immunohistochemistry in more than half of the cases (Fig. 5). Whether

this protein expression is due to a release of a post-transcriptional block or a suppression of their rapid degradation in normal epithelia is not known (Su *et al*., 1994). Another simple epithelia-associated keratin, K19, was shown to be present in the basal cell layer in normal non-cornified oral epithelia but not in cornified samples (Lindberg and Rheinwald, 1989). In moderate to severe dysplasia and carcinoma *in situ*, whether hyperkeratinized or not, strong staining for K19 was found in basal and suprabasal layers. Similar results were obtained in another study, although the staining for K19 was more heterogeneous in and between samples (Su *et al*., 1996). In this study, K19 mRNA expression was present in cornified and non-cornified normal epithelia but with a higher density of labeling in suprabasal cells of dysplastic epithelia. It is noteworthy that inflamed gingival epithelium exhibits diffuse staining for K19 (Bosch *et al*., 1989). Hence, it is difficult to evaluate the significance of K19-staining in epithelial dysplasias.

Loss of differentiation-related keratins in dysplastic lesions may be the most promising keratin-related marker of dysplasia and malignant development. Large-scale studies relating the changes in keratin expression to subsequent malignant development, however, are lacking.

(VI) Summary and Future Directions

Conventional clinical (subtype of leukoplakia) and histopathological (presence or absence of epithelial dysplasia) characteristics are still the most important parameters for the prediction of malignant transformation in oral pre-malignant lesions in routine diagnostic oral pathology. Thus, careful oral examination and a biopsy are usually required for optimal management to be determined. In particular, a non-homogeneous type of leukoplakia and the presence of distinct epithelial dysplasia are indications of a lesion at risk for malignant change. In general, the evidence for this latter statement is founded on selected patient materials, at least in the Western part of the world, and study designs have rarely controlled for possible confounders. This, in part, is due to the rather small numbers of patients enrolled in clinical studies, as well as to ethical considerations. Vivid discussions among specialists indicate a lack of consensus on the issues surrounding predictors of malignant transformation (Silverman *et al*., 1996; Ephros and Samit, 1997; Allen, 1997; McCartan, 1998; Mignogna *et al*., 2002; Zhang and Rosin, 2002). Collaborative, well-controlled clinico-pathologic studies, therefore, are needed if the generally accepted views are to be either substantiated or changed.

The use of molecular biological markers for predicting malignant transformation of oral pre-malignant lesions is intriguing and rapidly evolving. So far, these studies have not demonstrated methods that are readily applicable for routine diagnostic work. There is little doubt, however, that future developments will render these biological markers as valuable diagnostic tools.

In future studies, it may be important to evaluate the combined significance of several markers and/or clinical and histological variables for their prognostic value. Combinations of several molecular/genetic markers (Rosin *et al*., 2000), molecular/genetic markers and histopathology (Cruz *et al*., 1998; Lee *et al*., 2000; Zhang and Rosin, 2001), and clinical and histopathological characteristics (Schepman and van der Waal, 1995; van der Waal *et al*., 2000) have been proposed. Previously, examination of genetic events has occurred at the single-gene level; however, array technologies have made it possible for thousands of genes to be monitored simultaneously, making possible a better understanding of the events characterizing the different stages of cancer development (Patel *et al*., 2001). Classification and staging systems for oral leukoplakia need to be validated in future studies. Today, we still rely heavily on clinical judgments, adequate biopsies, and histopathological examinations.

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