Review

Update on Molecular Pathology in Oral Cancer and Precancer

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Abstract. *Oral carcinogenesis is a multifactorial process involving numerous genetic events that alter normal functions of oncogenes and tumour suppressor genes. This may increase the production of growth factors or the number of receptors on the cell surface, and/or increase transcription factors or intracellular signal messengers. Together with the loss of tumour suppressor activity, these changes lead to a cell phenotype that can increase cell proliferation, with loss of cell cohesion, and infiltration of adjacent tissue thus causing distant metastasis. Molecular pathology is responsible for defining the molecular mechanisms that underlie the onset of oral precancer and cancer. The aim of this review is to describe recent advances in our understanding of the molecular control of the innumerable pathways related to these processes. These may lead to short- or medium term improvements in the diagnosis and prognosis of oral precancerous and cancerous lesions and to the development of novel therapeutic approaches to this disease.*

Head and neck cancer (HNC) is the sixth most common human cancer (1), representing 3% of all types of cancer. They are located in the oral cavity in 48% of cases, and 90% of these are oral squamous cell carcinomas (OSCC) (2), which are sometimes preceded by precancerous lesions, *e.g.* leukoplakia, erythroplakia, actinic cheilitis and palatal keratosis associated with inverted smoking (3). An increase in

oral cancer prevalence among young adults is a cause of special concern. There has been a 60% increase in the number of under 40-year-olds with tongue cancer over the past 30 years. However, few data have been published on the aetiology and natural history of oral cancer. Because more than half of patients have not been exposed to the carcinogens usually implicated in the disease, *e.g.* alcohol or tobacco, other risk factors have been sought and proposed. These include genetic predisposition, immunodeficiency, diet and viral infections, *e.g.* HPV (human papillomavirus) and HHV (human herpes virus). Moreover, it has been claimed that these cases show a higher aggressiveness and worse prognosis (4).

Oral precancerous lesions are usually histologically classified by the presence or absence of oral epithelial dysplasia (OED), categorised by the WHO (2003) as slight, moderate, severe hyperplasia or carcinoma *in situ* according to the presence and severity of cell atypia and other structural aspects of the epithelium. This proposal was incorporated into a new WHO classification of head and neck tumours published in 2005 (5). This histopathological grading of OED was designed to assist prediction of the malignant transformation of these lesions. Nevertheless, no objective methods are yet available to typify dysplastic lesions and allow consistent and reproducible results to be obtained (6), since not all oral lesions with OED progress to OSCC. Moreover, the utility of this subdivision of OED has been questioned, since wide inter- and intra observer variability has been observed in assessments of the presence/absence and degree of this condition (7). A binary OED classification method was recently described, dividing them between lesions at low and high risk of malignant transformation. This could be used as a complementary method to improve the sensitivity and specificity offered by the 2005 system in current use (6).

Oral carcinogenesis is a highly complex multifactorial process that takes place when epithelial cells are affected

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by several genetic alterations. The use of molecular biology techniques to diagnose oral precancerous and cancerous lesions may markedly improve the detection of alterations that are invisible under the microscope. This would aid identification of patients at a higher risk of developing oral cancer (8). Despite advances in the treatment of oral cancer, the survival of these patients remains very low, mainly due to their high risk of developing a second cancer. (1).

This review presents up-to-date evidence on molecular markers that have shown promising results in predicting the progression of oral precancerous disease and evaluating the efficacy of oral cancer treatments, with the potential to markedly increase the patient survival rate. Reference is also made to studies of head and neck cancer when it is not specified whether or not they are OSCCs.

Cytogenetic and Epigenetic Alterations in Oral Cancer

Chromosomal instability. The most widely used molecular approach to the loss of genetic material is the study of loss of heterozygosity (LOH) based on the detection of differences between normal maternal or paternal alleles in constitutional (normal) DNA and their homonyms in tumour tissue. When a multicellular organism inherits a mutation in an allele of a tumour suppressor gene, it is considered a heterozygote of that gene, *i.e.* it possesses a mutated (mutated allele) and a normal (normal allele) copy. When a somatic cell with this inherited mutation has a second mutation in the other allele of the suppressor gene, they are both mutated and there is a complete loss of normal protein function, a lack of suppressor function. This is a case of LOH, since both alleles are mutated and the cell or cells that derive from them will therefore be homozygotes (both alleles mutated).

Alterations in the length of short repetitive sequences of DNA (one or two nucleotides) are described by the term 'microsatellite instability' (MSI). MSI results from the functional failure of DNA repair genes, which are designated 'mismatch repair' (MMR) genes. MSI can be detected by comparing the selective amplification of microsatellite loci in DNA from paraffin-embedded tumour samples with that in DNA from normal tissue. Alterations in these regions have been used as clonality markers and to detect tumour cells among normal cells (9, 10).

Various researchers using microsatellite markers demonstrated that alterations in certain regions of chromosomes 3p, 9p, 17p and 18q are related to the development of HNC (11). Numerous studies have identified specific genetic alterations in OSCCs and oral precancerous lesions (Table I). Bockmuhl *et al.*, using comparative

genomic hybridization, found deletions of chromosomes 3p, 5q and 9p with 3q gain in well-differentiated HNC *versus* deletions of 4q, 8p,11q,13q,18q and 21q and gains in 1p,11q,13,19 and 22q in poorly differentiated HNC, indicating a relationship with tumour progression (12).

Researchers using novel molecular techniques, *e.g.* microsatellite and restriction fragment length polymorphism (RFLP) tests, found the most common genetic change to be loss of the 9p21-22 chromosomal region (containing gene $p16^{INK4a}/p14^{ARF}/CDKN2A$), an early event in 70% of all HNCs (1, 11, 13). *P16* inhibits *CDK2* and *CDK6*, preventing initiation of the cell cycle and producing non-phosphorylation of *pRb*, causing cell cycle arrest in G1. El Naggar *et al.* found LOH in 9p21-p22 in 72% of HNCs in a preinvasive and/or invasive state (14).

Loss of the 3p region is another early cytogenetic change in oral carcinogenesis, with even benign hyperplastic lesions (the earliest precancerous lesions) showing loss of 9p21 or 3p in 30% of cases (13). Allelic losses in 13q and 8p are more frequent in OSCC than in OED (15).

Amplification of 11q13 has been observed in around one-third of head and neck cancers. This region contains the proto-oncogene cyclin *D1/PRAD1/CCD1*, whose function is to activate *pRb* by phosphorylation, facilitating progression of the cell cycle from the G1 (growth) to the S (synthesis) phase. Hence, D1 cyclin activation and p16^{INK4a} inactivation have the same effect, *i.e.*, an increase in *pRb* phosphorylation and progression from G1 to S in the cell cycle (13).

Loss of the 17p13 chromosomal region is considered a late effect in the progression of HNC. This region contains gene *p53*, which was mutated in 79% of 123 HNCs analysed by Balz *et al.* (16). Figure 1 depicts the genetic alterations most frequently observed during progression of these lesions.

A retrospective study by Mao *et al.* demonstrated that precancerous lesions with loss of 9p21 and 3p14 chromosomes are more prevalent in patients who subsequently develop a HNC (17). Another study of patients with benign or precancerous lesions with OED showed that those with losses of only 3p and 9p had a 3.8-fold higher risk of cancer progression, whereas those with losses of 4q, 8p, 11q and 17p had a 33-fold higher risk (18).

Gain of DNA content by aneuploidy may also be a predictor of cancer in precancerous lesions, since aneuploid oral leukoplakia lesions show higher rates of malignant progression to OSCC (13). A controversial study by Subdo *et al.* of 150 patients with dysplastic leukoplakias found that 70% were low-risk diploid lesions (3% progressed to OSCC), 13% were intermediate-risk tetraploid lesions (60% progressed to OSCC) and 17% were high-risk aneuploid lesions (84% progressed to OSCC). Nevertheless, these data must be verified by other research groups, since they were subsequently rejected by the editorial committee of the journal to which they were published (19).
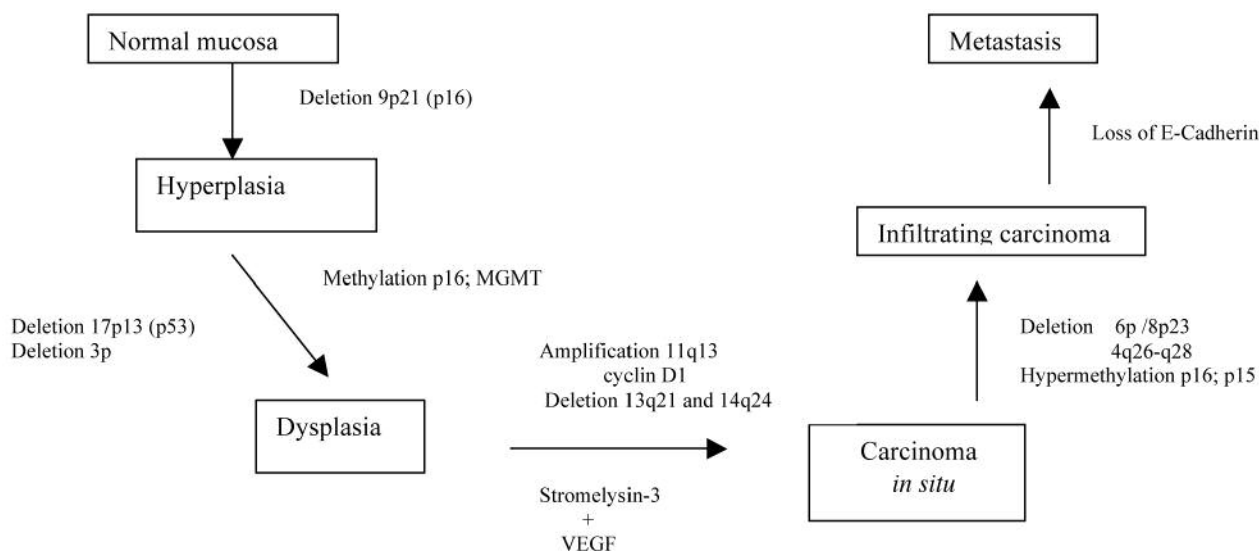
Figure 1. *Sequence of genetic and tumor/stroma alterations implicated in origin of oral cancer. [Modified from Califano, 1996 (11)].*

Partridge *et al*. identified significantly higher allelic imbalances in TNM stage 4 patients than in patients with stages 1-3. The mortality rate was 25-fold higher in patients with allelic imbalance at one or more loci of 3p24-26, 3p21, 3p13 and 9p21 than in patients with heterozygosity at these loci (20).

Another study reported that intraoral OEDs at sites carrying a high risk of developing OSCC (*e.g.*, floor of mouth, ventrolateral area of tongue and soft palate) had a higher LOH frequency and a loss pattern associated with an increased risk of malignant progression (21).

Epigenetic alterations. There is considerable interest in the role of epigenetic alterations in cancer, explaining their separate consideration in this review. These alterations affect or inactivate gene function by methylation of the promoter region, without changing the structure or sequence of the gene. Methylation is an epigenetic modification by which gene activity is controlled by addition of methyl groups (CH_3) to certain DNA cytosines (22). Most methylations occur in cytosines of CpG nucleotides (cytosine and guanine separated by a phosphate) and are present in a normal manner in promoter regions of certain genes. CpG islands act directly by inhibiting the binding of transcription factors to the DNA or by recruiting proteins that activate histone deacetylases (HDAC), which contain co-repressor complexes additional to DNA methylation. This phenomenon is the main epigenetic modification in humans and methylation pattern changes may play a very important role in carcinogenesis (23) because they are frequently related to the loss of gene expression. They also appear to be essential for the occurrence of the multiple genetic events required for

tumour progression, since they can inactivate DNA-repair genes. Both hypermethylation (by suppressor gene inactivation) and hypomethylation (by inappropriate oncogene activation) can produce carcinogenesis (24).

Rosas *et al*. (23) used a methylation-specific polymerase chain reaction (PCR) to study methylation patterns of cyclin-dependent kinase 2A/p16 (*CDKN2A/p16^{INK4a}*), methylguanine-DNA methyltransferase (*MGMT*) and death-associated protein kinase (DAP-K) genes in saliva of HNC patients, detecting abnormal hypermethylation patterns in all three. They reported that this PCR technique offers effective and sensitive tumour DNA detection and is potentially useful to detect and follow up recurrences in these patients.

Apparently histologically normal tissue adjacent to tumours (25-27) and precancerous lesions (28) may also have high levels of methylation of some genes, suggesting that methylation is an early event in oral carcinogenesis (Figure 1). It has been proposed that a more sensitive evaluation of tumour resection margins might be achieved by studying methylation than by determining histological alterations (29).

Although research into gene methylation in oral cavity tumours is still relatively recent, four important genes have already been analysed in detail: *CDKN2A/p16^{INK4a}*, *CDH1 (Cadherin-1), MGMT* and *DAPK1* (22, 30).

Gene methylation panels may be useful in oral cancer screening. Ogi *et al*. detected promoter methylation in at least 1 out of 12 genes in 67 out of 96 OSCCs (31). Viswanathan *et al*. used 5 hypermethylated genes (*p16^{INK4a}, p15^{INK4b}, human multL homolog 1 (hMLH1), MGMT* and *E-cadherin*) and detected OSCC in 38 out of 51 Indian patients (32). These studies show that a panel of specific markers can be designed

Table I. *Common genetic alterations in Head and Neck Carcinoma (HNC) (Modified from Kim and Califano, 2004).*

Location and/or gene	Type of alteration and frequency	Reference
9p21-22/ $p16^{INK4a}$/$p14^{ARF}$	Loss of heterozygosity (LOH) in 70%	Califano, 1996 (11); El Naggar, 1995 (14)
3p21/ RASSF1A	30% LOH in precancerous lesions	Kim and Califano, 2004 (13)
11q13/(PRAD-1/Cyclin D1/hst-1/int-2)	Amplification in >33% of HNCs	Kim and Califano, 2004 (13); Balz, 2003 (16)
9p21/p15	Hypermethylation in 30% of OSCCs	Shaw, 2006 (22)
9p21/p16 (CDKN2A)	Hypermethylation in 76% of OSCCs	Sanchez-Cespedes, 2000 (33); Ha and Califano, 2006 (30)
EGFR	Alteration of pathway by overexpression or other mechanism in 90%	Grandis, 1993 (41)
	Amplification in 30% of OSCCs	Scully, 1993 (42)
H-ras	Mutation in 55% of lip cancers	Milasin, 1994 (45)
	Mutation in 35% of OSCCs	Kuo, 1994 (46)
17p13/p53	Mutation in 79% of HNCs	Balz, 2003 (16)
	Overexpression in 47% of precancerous lesions	Warnakulasuriya, 1998 (7)
19q34/DAPK	Hypermethylation in 27% of sera from HNC patients	Sanchez-Cespedes, 2000 (33)
10q26/MGMT	Hypermethylation in 56% of saliva samples	López, 2003 (34)
	Hypermethylation in 7- 68% of primary tumours	Ha and Califano, 2006 (30)

HNC: Head and neck carcinoma; RASSF1A: Ras association domain family 1A.

to characterize and detect oral cavity tumours. In this context, *E-cadherin* hypermethylation has been associated with a more severe histological grade and worse survival rates (22).

Sánchez-Cespedes *et al.* were the first to use the methylation state as a clinical marker, using methylation-specific PCR to analyse 4 markers (*CDKN2A, MGMT, DAPK* and *glutathione S-transferase Pi gene (GSTP1)* in sera from 95 patients with primary HNC. At least one gene was methylated in 52 out of 95 patients and 42% of serum samples analysed (21/50) showed the corresponding methylation pattern; *DAPK* promoter hypermethylation was observed in 27% of cases. The authors proposed that this serum analysis may be a viable method to determine tumour recurrence in treated patients (33).

Rosas *et al.* studied saliva samples from 30 patients with HNC, using methylation-specific PCR with the markers *CDKN2A, MGMT* and *DAPK*. At least one gene was methylated in 17 out of the 30 primary tumour samples, and 11 of the 17 corresponding saliva samples showed the same methylation pattern. There appears to be a higher probability of tumour detection in the saliva of patients with oral cavity tumours than in the saliva of those with tumours distant from the aerodigestive tract (23).

López *et al.* used methylation-specific PCR to analyse the methylation state of genes $p16^{INK4a}$, $p14^{ARF}$ and *MGMT* in saliva samples from 34 patients with oral leukoplakia lesions; methylation of $p16^{INK4a}$, *MGMT* and $p14^{ARF}$ was observed in 44%, 56% and 1% of samples, respectively. These findings demonstrate that methylation is an early event in oral carcinogenesis and that its study may be useful to detect precancerous lesions (Figure 1). Screening studies of general populations are required to assess its applicability as a diagnostic method for suspicious oral lesions (34).

Hypermethylation of the cell cycle-regulating gene promoters $p16^{INK4a}$ and $p15^{INK4b}$ was found to be common in OSCC (*p16*: 76%; *p15*: 30%), although no significant correlation was observed with clinico-pathological characteristics or with the prognosis (22, 33).

To date, the rapid histopathological study of fresh tissue has been considered adequate for the intraoperative assessment of surgical safety margins. Some researchers have investigated the molecular analysis of margins but none have addressed its feasibility. Goldenberg *et al.*, using methylation-specific PCR, assessed margins during surgery by analysing the methylation of *CDKN2A* and *MGMT*. They concluded that this method can be intraoperatively applied and represents a new and feasible application of molecular analysis to the surgical treatment of oral cancer (35).

Hypermethylation can be reversed by means of agents such as 5-azacytidine (5AC). This treatment has proven to be clinically and biologically effective in malignant lung tumours and HNCs, associated with a decreased hypermethylation of $p15^{INK4b}$, or $p16^{INK4a}$ in the case of HNC. The main drawback is the toxicity of these agents (22). HDAC inhibitors have been used for haematological lesions and, in clinical trials, for solid tumours. In laboratory experiments, the combined application of 5AC and HDAC inhibitors has shown a promising synergistic effect (22).

Tumour–Stroma Interactions

Development by tumour cells of the ability to infiltrate surrounding tissue and produce metastasis is one of the final steps in tumour progression. This ability depends on destruction of the extracellular matrix (ECM), other stromal components

and the basement membrane. Matrix metalloproteinases (MMPs) form a family of zinc dependent endopeptidases that appear to play a major role in ECM degradation and in tumour invasion and metastasis (13). MMP-2, MMP-9 and MMP-3 have all been found to be related to tumour invasion, a more aggressive growth and a worse prognosis. Expression of these genes could be used as tumour progression marker (1).

Stromelysin 3, MMP11, was initially reported due to its overexpression in primary breast carcinoma. Its expression in hyperplastic and dysplastic oral lesions suggests its association with phenotypic alterations acquired early during the malignant transformation of oral epithelium (13). Soni *et al*. recently demonstrated that expression of stromelysin 3 is an early event in oral tumorigenesis (36). They immunohistochemically analysed stromelysin 3, erythroblastosis virus E26 oncogene homolog 1 (Ets-1) and vascular endothelial growth factor (VEGF) in 220 OSCCs, 90 precancerous lesions (59 hyperplasias and 31 dysplasias) and 81 normal oral mucosa samples. They found that the combined expression of stromelysin 3 and Ets-1 favours development of a precancerous state and that combined expression of stromelysin 3 and VEGF is related to progression from precancer to cancer (Figure 1). They concluded that VEGF expression is an adverse prognostic factor for patient survival in oral cancer (36).

E-cadherin is a 120 kDa transmembrane glycoprotein that participates in calcium-mediated cell adhesion. The loss or reduction of E-cadherin-mediated cell adhesion is an important step in the development of invasion and metastasis in numerous carcinomas, including OSCCs (37). Santos-García *et al*. demonstrated that loss of E-cadherin expression is an early event, being detected in moderate dysplasias, and is greater in carcinomas *in situ* and microinvasive carcinomas, suggesting that loss of epithelial cohesion may be an indicator of progression in these lesions (Figure 1). Thus, a marked and similar loss of expression was observed in OSCCs and lymph-node metastases, which increased with progression of the disease (38).

Another important mechanism for acquiring an invasive phenotype is anoikis resistance, defined as the ability to escape apoptosis by cell separation from the basement membrane, cell morphology changes and keratin accumulation. Swan *et al*. found that acquisition of anoikis resistance may be a prerequisite for the development of an invasive tumour phenotype and implies a worse prognosis in oral cancer (39).

Kupferman *et al*. recently used real-time RT-PCR to establish differences in the expression of genes *S100P, KLK6* and *CTNNAL1* between anoikis-resistant and anoikis-sensitive cell lines. The anoikis-resistant phenotype of OSCC has a distinct gene expression pattern, marked by chromosomal alterations that may contribute towards a differential expression of genes involved in various cellular functions. These mediators of anoikis resistance may serve as therapeutic target in cases of OSCC metastasis (40).

Oncogenes

Oncogenes are growth regulating and promoting genes that control transduction pathways of cell signals. Therefore, a mutation of these genes can lead to an overproduction or an increase in the function of the stimulatory proteins. Although isolated oncogenes are not able to transform normal epithelial cells, they appear to be important initiators of the process and are known to produce cell changes with the mutation of one copy of the gene (9).

Numerous oncogenes have been implicated in oral carcinogenesis. Aberrant expression of proto-oncogene epidermal growth factor receptor, (EGFR/c-erb 1) and members of gene families *ras, c-myc, int-2/Fgf-3 (fibroblast growth factor-3), hst-1/HSTF1 (heparin-binding secretory transforming factor 1), PRAD-1 (parathyroid adenomatosis 1), and bcl-1(B-cell leukemia/lymphoma 1)* is believed to contribute to cancer development (1).

Aberrant expression of transforming growth factor-α (TGF-α) is usually an early event in carcinogenesis. It is first observed in hyperplastic epithelium and then in OSCC, in the infiltrate of inflammatory cells around the infiltrating epithelium, especially eosinophils. TGF-α stimulates cell proliferation by autocrine and paracrine binding to EGFR (1). It is also believed to stimulate angiogenesis and has been described in 'normal' oral mucosa of patients that subsequently develop a second primary (41). EGFR, the biological receptor of EGF and TGF-α, is frequently overexpressed in oral cancer (1) and was shown to result from EGFR gene amplification in 30% of oral cancers (42).

Epidermal growth factor (EGF) influences cell division, migration, adhesion, differentiation and apoptosis *via* the tyrosine-kinase pathway. Elevated expression of EGF is a predictor of recurrence and a worse prognosis in HNC (13). Expression of its transmembrane receptor (EGFR) has been detected by immunohistochemistry (IHC) in 40-80% of OSCC cases and has been proposed to have prognostic value in these patients (43). Numerous studies have found that EGFR has a negative prognostic value, with its expression implying a higher likelihood of post-treatment recurrence and worse survival (41).

An ICH study by Ralhan *et al*. found a lower expression of retinoid receptors RARβ, γ and RXRα and of cell cycle regulators p16 and p21 in most of 244 OSCCs and 102 potentially malignant lesions than in normal oral mucosa samples. In the multivariate analysis, phenotype RAR α^+/p21$^-$ (+, RAR α accumulation; $-$, loss of p21) was related to a lower survival rate, demonstrating the prognostic value of the expression of retinoid receptors and their interaction with cell cycle regulators in oral tumorigenesis (44).

Some studies showed that members of the *ras* oncogene family are overexpressed in oral cancer. Although loss of control of *N-ras* may be an early step in oral carcinogenesis, with

increased expression in dysplastic lesions, *ras* mutations are infrequent in Western patients and detected in fewer than 5% of oral cancers (1). In contrast, 55% of lip cancers have *H-ras* mutation (45), which is also present in 35% of oral cancers in Asian populations in association with betel nut chewing (46).

Transcription factors that activate other genes are also activated in oral cancer. We highlight *c-myc*, which participates in the regulation of cell proliferation. This gene is frequently overexpressed in OSCC as a result of gene amplification, which is itself usually related to poorly differentiated tumours (1). *C-Myc* induces cell proliferation and, in combination with *p53*, apoptosis. Nuclear protein pRb of the *pRb* gene interacts with the c-myc gene, hindering its transcription and thereby inhibiting cell proliferation.

The gene *PRAD-1(cyclin D1/Bcl-1)* is located in 11q13 and encodes cyclin D, which, together with the product of the pRb gene, controls cell cycle transition from the G1 to the S phase. This gene is amplified in 30-50% of HNCs. Amplification of *PRAD-1* has been correlated with cytological grade, infiltrative growth pattern and metastasis (1).

Researchers using fluorescent *in situ* hybridization (FISH) with tissue microarray (TMA) found different oncogene amplification patterns according to the localization of HNCs, with more frequent amplification of *CCND1* in pharyngeal carcinomas and of *ZNF217* in oral and laryngeal carcinomas. They concluded that their methodology was effective to assess molecular changes and their relevance for HNC aetiology and progression (47).

For Kuo *et al.*, the classification of OSCC by gene expression is a future possibility. Using a microarray that analysed 4,324 genes, they reported 210 genes possibly related to oral cancer. They identified several genes with altered expression (*e.g. CKS1, TSPY, CBK, TLE4* and *BCHE*) that had not previously been related to OSCC, although they had been associated with other types of cancer (46, 48).

Méndez *et al.* reported 239 overexpressed and 75 underexpressed genes in OSCC. However, they found no difference in gene expression between incipient and advanced stages, or between tumours with and without metastasis (49). In the future, we shall be able to use these gene expression patterns to obtain a unique molecular 'fingerprint' for each tumour (50)

Finally, amplification of *int-2*, also known as fibroblast growth factor-3 (*Fgf-3*), which stimulates proliferation of mesenchymal, epithelial and neuroectodermal cells, has been described in premalignant dysplastic and hyperplastic lesions of the oral cavity. This suggests that *int-2* may be amplified before development of OSCC (1).

Tumour Suppressor Genes

Although oncogenes alone are not able to cause oral cancer, they appear to be initiators of the process. The crucial event in the transformation from premalignancy to malignancy is inactivation of negative cell regulators (tumour suppressor genes). Tumour suppressor genes are frequently inactivated by point mutations, deletions and rearrangements in both copies of the gene (1, 51).

Among the most widely studied tumour suppressor genes are *rb* and *p53*, which express pRb (retinoblastoma protein) and p53 protein respectively. These proteins control the cell cycle and are involved in the inhibition of cell proliferation. Mutations in these proteins produce uncontrolled cell proliferation. P53 acts as transcription factor of cell cycle inhibitors such as $p21^{Waf1/Cip1/Sdi1}$ and prevents the cell from going beyond phase G1 of the cell cycle, permitting DNA repair. If this is not possible, p53 induces apoptosis of these cells to avoid the transmission of potentially carcinogenic information. The *p53* gene is inactivated in approximately half of HNC cases but there is debate about the timing of this change in oral carcinogenesis. Aberrant expression of *p53* has been reported in mild dysplasias by some authors but in association with advanced tumours by others (11). Overexpression of protein p53 and gene mutation have also been detected in OED adjacent to OSCC lesions (1, 11, 52).

A meta-analysis of results published in seven studies showed that a much higher percentage (47%) of premalignant oral lesions had p53 overexpression than underwent malignant transformation (7). However, another study proposed that p53 expression above the basal layer of the epithelium indicates carcinoma development, even in the absence of epithelial dysplasia (53, 54). Nonetheless, given that p53 positivity is not observed in all lesions that undergo malignant transformation, it is recommended to take into account conventional histological parameters or introduce new markers that are currently under study.

Lee *et al.* found a relationship between p53 positivity and a higher risk of oral cancer, especially when combined with chromosome polysomy and LOH in chromosome 3p or 9p (54). Partridge *et al.* looked for *p53* mutations, allelic imbalance of protein p53 and expression of mdm2 in 45 cases of OSCC and found allelic imbalance in 23% and *p53* mutation in 33% of cases. This discrepancy may suggest that normal and mutated p53 coexist in the same tumour and may be explained by the presence of oncogenic human papillomavirus (HPV) in 10-42% of OSCCs without *p53* mutation (55).

Another suppressor gene is *INK4-ARF*, whose product, p16 protein, acts as powerful inhibitor of cyclin-dependent kinases (CDK4 and CDK6). Thus, inhibition of this factor, along with overexpression of cyclin D1, increases CDK4/6 activity and stimulates cell proliferation (56). Conversely, replacement of p26 in OSCC tumour cells had significant anti tumour effects in culture tissues and animal models (57).

Absence of pRb expression was observed in 66% of OSCCs and 64% of premalignant lesions, and absence of p16 expression in 63% of OSCCs and 59% of premalignant lesions (58).

Using IHC in 220 OSCC samples and 90 premalignant lesions (38 with dysplasia), Soni *et al*. found dysregulation of the p16/pRb/D1 cyclin pathway to be an initial event in the onset of epithelial dysplasia. Thus, 90% of OSCCs and 83% of premalignant lesions showed altered expression of some protein of the pRb pathway. Moreover, simultaneous alteration of the pRb pathway and p53 was related to malignant transformation and a worse prognosis (59).

Viruses

Oncogenic viruses represent another factor that may affect cell cycle regulation. There is increasing interest in the role of infection by HPV in HNC. A meta-analysis by Miller and Johnstone found the probability of detecting HPV to be 2 to 3-fold higher in precancerous oral mucosa and 4 to 5-fold higher in OSCC than in normal oral epithelium. They also reported a frequent association between high-risk HPVs (HPV 16 and 18) and OSCC lesions (60). A recent systematic review of 5,046 samples of head and neck squamous cell carcinoma (HNSCC) in 60 PCR studies showed a significantly higher prevalence of HPV in cancer of the oropharynx than in that of the oral cavity (35.6% *vs*. 23.5%). The most prevalent genotype at both sites was HPV 16, with a more frequent presence of HPV 18 in oral cavity than oropharyngeal lesions (61).

Oncogenic transformation by these HPVs depends on oncoproteins E6 and E7, which bind with wild-type p53 and pRb proteins and remove their ability to stimulate DNA-repair or apoptosis. This inactivation of tumour suppressor proteins represents a possible pathogenic mechanism (42).

Mishra *et al*. recently reported a higher expression of transcription factor NF-κB and its activation sequences in oral cancer that arises from OED lesions. Thus, HPV infection would activate NF-κB-p50/p65 complexes, which would in turn stimulate differentiation of oral neoplastic cells, improving the prognosis. However, the authors acknowledged that further research is required to confirm and explain this finding (62).

As occurred with the development of a vaccine to prevent HPV-related cervical cancer, animal experiments are under way to develop a similar vaccine for OSCC. Results to date suggest that L1-based immunization may prevent the development of OSCC associated with HPV (63).

Conclusion

Despite major advances in the molecular pathology of HNC and oral cancer, there remain numerous gaps in our knowledge of the molecular markers involved in oral carcinogenesis.

OSCCs appear to have a multifocal character, with only half of them developing on the same site as a previous leukoplakia. Complex molecular mechanisms are implicated

and the identification of a single marker to predict outcomes in all oral premalignant lesions remains a difficult challenge. Among reported markers are those for oncogene activation, *e.g. ras, EGFR, PRAD-1, int-2,* and for inactivation of tumour suppressor genes, notably *p53, pRb* and *INK4-ARF*. Their study may contribute to the search for reliable molecular markers of the development of oral cancer.

Other molecular pathology approaches are emerging, including assessment of the hypermethylation of the promoter regions of certain genes (*e.g. p16^{INK4a}, MGMT, DAPK1*) or tumour-stroma interactions, which could provide new and promising data. However, further research is essential to clarify the role and clinical significance of these molecular changes, allowing them to be used as effective diagnostic and prognostic markers to improve the management of these patients.

References

1 Williams HK: Molecular pathogenesis of oral squamous carcinoma. Mol Pathol *53*: 165-172, 2000.

2 Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ and Thun MJ: Cancer statistics, 2004. CA Cancer J Clin *54*: 8-29, 2004.

3 Pindborg JJ, Reichart PA, Smith CJ, Van der Waal I, and in collaboration with Sobin LH and pathologists in 9 countries: Histological-typing of Cancer and Precancer of the Oral Mucosa. New York, Springer Verlag, 1997.

4 Schantz SP and Yu GP: Head and neck cancer incidence trends in young Americans, 1973-1997, with a special analysis for tongue cancer. Arch Otolaryngol Head Neck Surg *128*: 268-274, 2002.

5 Barnes L, Eveson JW, Reichart PA and Sidransky D: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Head and Neck. Lyon: IARC-press, 2005.

6 Kujan O, Oliver RJ, Khattab A, Roberts SA, Thakker N and Sloan P: Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. Oral Oncol *42*: 987-993, 2006.

7 Warnakulasuriya KA, Tavassoli M and Johnson NW: Relationship of p53 overexpression to other cell cycle regulatory proteins in oral squamous cell carcinoma. J Oral Pathol Med *27*: 376-381, 1998.

8 Joseph BK: Oral cancer: prevention and detection. Med Princ Pract *11(Suppl 1)*: 32-35, 2002.

9 Sidransky D: Molecular markers in cancer diagnosis. J Natl Cancer Inst Monogr 27-29, 1995.

10 Reshmi SC and Gollin SM: Chromosomal instability in oral cancer cells. J Dent Res *84*: 107-117, 2005.

11 Califano J, van der RP, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W and Sidransky D: Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res *56*: 2488-2492, 1996.

12 Bockmuhl U, Wolf G, Schmidt S, Schwendel A, Jahnke V, Dietel M and Petersen I: Genomic alterations associated with malignancy in head and neck cancer. Head Neck *20*: 145-151, 1998.

13 Kim MM and Califano JA: Molecular pathology of head-and-neck cancer. Int J Cancer 112: 545-553, 2004.

14 El Naggar AK, Hurr K, Batsakis JG, Luna MA, Goepfert H and Huff V: Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous carcinoma. Cancer Res 55: 2656-2659, 1995.

15 Braakhuis BJ, Leemans CR and Brakenhoff RH: A genetic progression model of oral cancer: current evidence and clinical implications. J Oral Pathol Med 33: 317-322, 2004.

16 Balz V, Scheckenbach K, Gotte K, Bockmuhl U, Petersen I and Bier H: Is the p53 inactivation frequency in squamous cell carcinomas of the head and neck underestimated? Analysis of p53 exons 2-11 and human papillomavirus 16/18 E6 transcripts in 123 unselected tumor specimens. Cancer Res 63: 1188-1191, 2003.

17 Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F and Sidransky D: Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 91: 9871-9875, 1994.

18 Rosin MP, Cheng X, Poh C, Lam WL, Huang Y, Lovas J, Berean K, Epstein JB, Priddy R, Le ND and Zhang L: Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. Clin Cancer Res 6: 357-362, 2000.

19 Curfman GD, Morrissey S and Drazen JM: Retraction: Sudbo J et al: DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med 344: 1270-1278, 2001 and Sudbo J et al: The influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J Med 350: 1405-13, 2004. N Engl J Med 355: 1927, 2006.

20 Partridge M, Emilion G, Pateromichelakis S, A'Hern R, Lee G, Phillips E, and Langdon J: The prognostic significance of allelic imbalance at key chromosomal loci in oral cancer. Br J Cancer 79: 1821-1827, 1999.

21 Zhang L, Cheung KJ, Jr., Lam WL, Cheng X, Poh C, Priddy R, Epstein J, Le ND and Rosin MP: Increased genetic damage in oral leukoplakia from high risk sites: potential impact on staging and clinical management. Cancer 91: 2148-2155, 2001.

22 Shaw R: The epigenetics of oral cancer. Int J Oral Maxillofac Surg 35: 101-108, 2006.

23 Rosas SL, Koch W, Costa Carvalho MG, Wu L, Califano J, Westra W, Jen J and Sidransky D: Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res 61: 939-942, 2001.

24 Auerkari EI: Methylation of tumor suppressor genes p16(INK4a), p27(Kip1) and E-cadherin in carcinogenesis. Oral Oncol 42: 5-13, 2006.

25 Kulkarni V and Saranath D: Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. Oral Oncol 40: 145-153, 2004.

26 Maruya S, Issa JP, Weber RS, Rosenthal DI, Haviland JC, Lotan R and El Naggar AK: Differential methylation status of tumor-associated genes in head and neck squamous carcinoma: incidence and potential implications. Clin Cancer Res 10: 3825-3830, 2004.

27 Yeh KT, Shih MC, Lin TH, Chen JC, Chang JY, Kao CF, Lin KL and Chang JG: The correlation between CpG methylation on promoter and protein expression of E-cadherin in oral squamous cell carcinoma. Anticancer Res 22: 3971-3975, 2002.

28 Kresty LA, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC and Weghorst CM: Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. Cancer Res 62: 5295-5300, 2002.

29 von Zeidler SV, Miracca EC, Nagai MA and Birman EG: Hypermethylation of the p16 gene in normal oral mucosa of smokers. Int J Mol Med 14: 807-811, 2004.

30 Ha PK and Califano JA: Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. Lancet Oncol 7: 77-82, 2006.

31 Ogi K, Toyota M, Ohe-Toyota M, Tanaka N, Noguchi M, Sonoda T, Kohama G and Tokino T: Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin Cancer Res 8: 3164-3171, 2002.

32 Viswanathan M, Tsuchida N and Shanmugam G: Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 105: 41-46, 2003.

33 Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG and Sidransky D: Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 60: 892-895, 2000.

34 Lopez M, Aguirre JM, Cuevas N, Anzola M, Videgain J, Aguirregaviria J and Martinez DP: Gene promoter hypermethylation in oral rinses of leukoplakia patients-a diagnostic and/or prognostic tool? Eur J Cancer 39: 2306-2309, 2003.

35 Goldenberg D, Harden S, Masayesva BG, Ha P, Benoit N, Westra WH, Koch WM, Sidransky D and Califano JA: Intraoperative molecular margin analysis in head and neck cancer. Arch Otolaryngol Head Neck Surg 130: 39-44, 2004.

36 Soni S, Mathur M, Shukla NK, Deo SV and Ralhan R: Stromelysin-3 expression is an early event in human oral tumorigenesis. Int J Cancer 107: 309-316, 2003.

37 Chow V, Yuen AP, Lam KY, Tsao GS, Ho WK and Wei WI: A comparative study of the clinicopathological significance of E-cadherin and catenins (alpha, beta, gamma) expression in the surgical management of oral tongue carcinoma. J Cancer Res Clin Oncol 127: 59-63, 2001.

38 Santos-Garcia A, Abad-Hernandez MM, Fonseca-Sanchez E, Julian-Gonzalez R, Galindo-Villardon P, Cruz-Hernandez JJ and Bullon-Sopelana A: E-cadherin, laminin and collagen IV expression in the evolution from dysplasia to oral squamous cell carcinoma. Med Oral Patol Oral Cir Bucal 11: E100-E105, 2006.

39 Swan EA, Jasser SA, Holsinger FC, Doan D, Bucana C and Myers JN: Acquisition of anoikis resistance is a critical step in the progression of oral tongue cancer. Oral Oncol 39: 648-655, 2003.

40 Kupferman ME, Patel V, Sriuranpong V, Amornphimoltham P, Jasser SA, Mandal M, Zhou G, Wang J, Coombes K, Multani A, Pathak S, Silvio GJ and Myers JN: Molecular analysis of anoikis resistance in oral cavity squamous cell carcinoma. Oral Oncol 43: 440-54, 2007.

41 Grandis JR and Tweardy DJ: Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 53: 3579-3584, 1993.

42 Scully C: Oncogenes, tumor suppressors and viruses in oral squamous carcinoma. J Oral Pathol Med 22: 337-347, 1993.

43 Altuna MX, Algaba GJ, Wang RJ, Weisman R and Ongkeko W: Immunohistochemistry study of EGFR expression in head and neck squamous cell carcinoma. Acta Otorrinolaringol Esp *56*: 143-146, 2005 (In Spanish).

44 Ralhan R, Chakravarti N, Kaur J, Sharma C, Kumar A, Mathur M, Bahadur S, Shukla NK and Deo SV: Clinical significance of altered expression of retinoid receptors in oral precancerous and cancerous lesions: relationship with cell cycle regulators. Int J Cancer *118*: 1077-1089, 2006.

45 Milasin J, Pujic N, Dedovic N, Nikolic Z, Petrovic V and Dimitrijevic B: High incidence of *H-ras* oncogene mutations in squamous cell carcinoma of lip vermilion. J Oral Pathol Med *23*: 298-301, 1994.

46 Kuo MY, Jeng JH, Chiang CP and Hahn LJ: Mutations of *Ki-ras* oncogene codon 12 in betel quid chewing-related human oral squamous cell carcinoma in Taiwan. J Oral Pathol Med *23*: 70-74, 1994.

47 Freier K, Joos S, Flechtenmacher C, Devens F, Benner A, Bosch FX, Lichter P and Hofele C: Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma. Cancer Res *63*: 1179-1182, 2003.

48 Kuo WP, Whipple ME, Sonis ST, Ohno-Machado L and Jenssen TK: Gene expression profiling by DNA microarrays and its application to dental research. Oral Oncol *38*: 650-656, 2002.

49 Mendez E, Cheng C, Farwell DG, Ricks S, Agoff SN, Futran ND, Weymuller EA Jr, Maronian NC, Zhao LP and Chen C: Transcriptional expression profiles of oral squamous cell carcinomas. Cancer *95*: 1482-1494, 2002.

50 Otero-Rey E, Garcia-Garcia A, Barros-Angueira F, Torres-Espanol M, Gandara-Rey JM and Somoza-Martin M: DNA microarrays in oral cancer. Med Oral *9*: 288-292, 2004.

51 Bascones-Ilundain C, Gonzalez-Moles M, Campo-Trapero J, Gil-Montoya J, Esparza-Gomez G, Cano-Sanchez J and Bascones-Martinez A: No differences in caspase-3 and Bax expression in atrophic-erosive *vs.* reticular oral lichen planus. J Eur Acad Dermatol Venereol *22*: 204-212, 2008.

52 Reibel J: Prognosis of oral pre-malignant lesions: significance of clinical, histopathological, and molecular biological characteristics. Crit Rev Oral Biol Med *14*: 47-62, 2003.

53 Cruz IB, Snijders PJ, Meijer CJ, Braakhuis BJ, Snow GB, Walboomers JM and van Der Waal I: p53 expression above the basal cell layer in oral mucosa is an early event of malignant transformation and has predictive value for developing oral squamous cell carcinoma. J Pathol *184*: 360-368, 1998.

54 Lee JJ, Hong WK, Hittelman WN, Mao L, Lotan R, Shin DM, Benner SE, Xu XC, Lee JS, Papadimitrakopoulou VM, Geyer C, Perez C, Martin JW, El Naggar AK and Lippman SM: Predicting cancer development in oral leukoplakia: ten years of translational research. Clin Cancer Res *6*: 1702-1710, 2000.

55 Partridge M, Kiguwa S, Emilion G, Pateromichelakis S, A'Hern R and Langdon JD: New insights into p53 protein stabilisation in oral squamous cell carcinoma. Oral Oncol *35*: 45-55, 1999.

56 Gonzalez-Moles MA, Gil-Montoya JA, Ruiz-Avila I, Esteban F, Delgado-Rodriguez M and Bascones-Martinez A: Prognostic significance of *p21^{WAF1/CIP1}*, *p16^{INK4a}* and *CD44s* in tongue cancer. Oncol Rep *18*: 389-396, 2007.

57 Rocco JW, Li D, Liggett WH Jr, Duan L, Saunders JK Jr, Sidransky D and O'Malley BW Jr: *p16^{INK4A}* adenovirus-mediated gene therapy for human head and neck squamous cell cancer. Clin Cancer Res *4*: 1697-1704, 1998.

58 Pande P, Mathur M, Shukla NK and Ralhan R: pRb and p16 protein alterations in human oral tumorigenesis. Oral Oncol *34*: 396-403, 1998.

59 Soni S, Kaur J, Kumar A, Chakravarti N, Mathur M, Bahadur S, Shukla NK, Deo SV and Ralhan R: Alterations of rb pathway components are frequent events in patients with oral epithelial dysplasia and predict clinical outcome in patients with squamous cell carcinoma. Oncology *68*: 314-325, 2005.

60 Miller CS and Johnstone BM: Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982-1997. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *91*: 622-635, 2001.

61 Kreimer AR, Clifford GM, Boyle P and Franceschi S: Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev *14*: 467-475, 2005.

62 Mishra A, Bharti AC, Varghese P, Saluja D and Das BC: Differential expression and activation of NF-kappaB family proteins during oral carcinogenesis: Role of high risk human papillomavirus infection. Int J Cancer *119*: 2840-2850, 2006.

63 Maeda H, Kubo K, Sugita Y, Miyamoto Y, Komatsu S, Takeuchi S, Umebayashi T, Morikawa S, Kawanishi K and Kameyama Y: DNA vaccine against hamster oral papillomavirus-associated oral cancer. J Int Med Res *33*: 647-653, 2005.