Abstract. There is considerable interest in the analysis of epigenetic alterations in cancer, including oral cancer and pre-cancerous lesions. These processes affect or inactivate the functions of genes without altering their structure or sequence. One example is the methylation of the promoter region of some genes involved in cell cycle control. Knowledge of methylation patterns is very important for understanding the expression of genes in normal and pathological situations. This review provides an update on research into this issue in oral cancer and pre-cancerous lesions. A greater understanding of this epigenetic alteration could not only assist the diagnosis and prognosis of oral cancer but could also open up novel therapeutic approaches. The presence of methylation in specific tumour suppressor genes could modify their function and alter cell cycle control, so the patients could have an increased risk of developing cancer and also a higher degree of malignancy. The most frequently and extensively studied methylated genes in oral premalignant lesions are p16, MGMT, RARβ2, E-cadherin, and DAP-kinase.

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1. Introduction
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1. Introduction

Cancer is the second cause of mortality in developed countries. Head and neck cancer represents 5-10% of cases in Europe, where there is a rising incidence of oral cancer and associated mortality, especially among young populations (1). The most frequent oral cancers are oral squamous cell carcinomas (OSCCs) which are the most malignant tumours of the head and neck (2). This aggressive epithelial neoplasm is associated with severe morbidity and >50% long-term survival, despite advances in surgical treatments and in radio- and chemotherapy.

These cancers are occasionally preceded by potentially malignant (pre-cancerous) lesions, such as leukoplakia and erythroplakia. Epidemiological data from different countries over the past 30 years have shown prevalence of oral leukoplakia ranging from 1.1 to 11.7% (mean of 2.9%), with prevalence among smokers ranging from 3.7 to 60.3% (3). Up to 10% of patients with leukoplakia have an invasive carcinoma (4), and the short survival of oral cancer on leukoplasias (only 30–40% survival at 5 years post-diagnosis) makes prevention of this malignant transformation essential (5).

Tobacco (including smoked and chewed variants) is the prime cause of oral cancer worldwide and is estimated to be responsible for around 400,000 new cases every year. Betel nut chewing has been implicated in OSCC, leukoplakia, and oral submucous fibrosis (progressive inflammation and fibrosis of connective tissue) (6). Other predisposing factors for oral cancer include alcohol use, gender, age and infection by human papillomavirus (7,8).

2. Concept of epigenetic alterations

Carcinogenesis is a multistep process in which cells accumulate changes in their genetic material, giving rise to an alteration of cell function. Some of these changes can not be attributed to a DNA sequence modification (e.g. deletion or mutation) and are designated epigenetic changes. These are
Table I. Genes whose methylation is related to head and neck cancer.

<table>
<thead>
<tr>
<th>Type of gene</th>
<th>Function</th>
<th>Action mechanism</th>
<th>% Methylation in tumour lesion/pre-cancerous lesion</th>
<th>% Methylation in saliva/healthy mucosa</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16&lt;sup&gt;INK4a&lt;/sup&gt; Tumour suppressor gene (regulates cell cycle)</td>
<td>Vital in Rb-cyclin D pathway to maintain retinoblastoma protein</td>
<td>27/31 (HNC)</td>
<td>18/10 (HNC)</td>
<td>31/NR, 79/NR</td>
<td>Sanchez-Cespedes, et al (16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47/31 (HNC)</td>
<td>32.5/79 (HNC)</td>
<td>31/NR, NR/NR</td>
<td>Rosas, et al (18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35/NR (severe dysplasia)</td>
<td>87.5/NR</td>
<td>87.5/NR, Viet, et al (19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23/NR (HNC)</td>
<td>NR/50 (oral leukoplasia)</td>
<td>50/NR, Rosas, et al (18)</td>
<td></td>
</tr>
<tr>
<td>DAP-kinase Tumour suppressor gene</td>
<td>Involved in apoptosis. Inhibits appearance of metastases</td>
<td>18/18 (HNC)</td>
<td>18/18 (HNC)</td>
<td>18/NR, Sanchez-Cespedes, et al (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>33/60 (HNC)</td>
<td>33/60 (HNC)</td>
<td>0/0, Rosas, et al (18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.8/60 (HNC)</td>
<td>NR/NR, 23.8/60 (HNC)</td>
<td>NR/NR, Hasegawa, et al (7)</td>
<td></td>
</tr>
<tr>
<td>E-cadherin Related to lymph node metastasis and cell invasion</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; dependent molecule. Regulates intercellular junctions</td>
<td>46/79 (HNC)</td>
<td>46/79 (OSSC)</td>
<td>46/79 (OSSC), Shaw (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/79 (OSCC)</td>
<td>7/79 (OSCC)</td>
<td>7/79 (OSCC), Shaw (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>85.4/79 (OSCC)</td>
<td>NR/33.3</td>
<td>85.4/79 (OSCC), Lee, et al (29)</td>
<td></td>
</tr>
<tr>
<td>RASSF1A Tumour suppressor gene (ras association domain family 1A)</td>
<td>Ras modulation</td>
<td>7.5/7.5 (HNC)</td>
<td>35/7.5 (salivary gland c.)</td>
<td>NR/NR, 35/7.5 (salivary gland c.)</td>
<td>Hasegawa, et al (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/7.5 (HNC)</td>
<td>NR/NR, 50/7.5 (HNC)</td>
<td>NR/NR, Lee, et al (29)</td>
<td></td>
</tr>
<tr>
<td>CLDN3 Regulates close intercellular junctions</td>
<td>Related to apoptosis mechanisms</td>
<td>69/69 (osseophageal c.)</td>
<td>NR/0</td>
<td>NR/0, Roth, et al (23)</td>
<td></td>
</tr>
<tr>
<td>CRBP Apoacelluar retinol-binding protein</td>
<td>Participates in binding of retinoic acid with its receptor</td>
<td>31/29 (osseophageal c.)</td>
<td>NR/0</td>
<td>NR/0, Roth, et al (23)</td>
<td></td>
</tr>
<tr>
<td>MT1G Involved in metabolism of metals and detoxifying mechanisms</td>
<td>Associated with hepatic storage of copper</td>
<td>62/57 (osseophageal c.)</td>
<td>NR/9</td>
<td>NR/9, Roth, et al (23)</td>
<td></td>
</tr>
</tbody>
</table>
Table I. Continued.

<table>
<thead>
<tr>
<th>Type of gene</th>
<th>Function</th>
<th>Action mechanism</th>
<th>% Methylation in tumour lesion/ pre-cancerous lesion</th>
<th>% Methylation in saliva/ healthy mucosa</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRTFDC1</td>
<td>Tumour suppressor gene (phosphoribosyl transferase domain containing 1)</td>
<td>Associated with pharmaceutical metabolism</td>
<td>17/NR (OSCC)</td>
<td>NR/NR</td>
<td>Suzuki, et al (34)</td>
</tr>
</tbody>
</table>

NR, not recorded. HNC, head and neck cancer; OSCC, oral squamous cell carcinoma. 'López, et al (25), 82% of patients were positive for the methylation of at least one of the studied genes in saliva samples, but the percentage corresponding to the methylation of each gene is not specified. ²Peritumoural healthy mucosa.

A positive result in serum DNA (loss of heterogeneity or presence of new alleles) was also reported to be a predictive factor for distant metastases, based on findings that were statistically significant despite a small sample size (20). In oral medicine, methylation has been analyzed in various types of sample: in head and neck tumours, focussing on the oral region and especially OSCC; in pre-cancerous lesions such as leukoplasia, lichen planus and different grades of dysplasia; and in healthy saliva and mucosa samples (to be continued).
establish comparisons with disease states). Sample sizes have been highly variable. In the present review, we found study populations of 95 (16) or 80 patients with primary head and neck tumours (7) and others of only 14 patients with OSCC or severe dysplasia (19). Sample sizes are smaller in studies of sera or pre-cancerous lesions than in studies of tumours. These discrepancies in sample sizes and in the different media analyzed hamper comparisons among results and the confirmation of a clear relationship between methylation and carcinogenesis.

Hypermethylation has been investigated in numerous genes in an attempt to establish a clear link between this epigenetic mechanism and carcinogenesis. Certain genes appear to be of special importance in this respect, although results have varied widely in some cases and depending on the type of study (Table I).

*p16INK4a*. This tumour suppressor gene inhibits kinase-dependent cyclines and is vital for keeping the retinoblastoma protein in its active non-phosphorylated state. Various studies have reported 27-76% methylation of this gene in tumours versus the 0% obtained in healthy oral mucosa (7,16,18,19,21,22). Interestingly, one study detected 72.73% methylation (21) in 22 samples of healthy peritumoural tissue, suggesting that p16 methylation may be an early event in carcinogenesis and might therefore serve as a prognostic and diagnostic marker. In studies on pre-cancerous lesions, it was reported that p16 methylation was not related to the malignant transformation of lichen planus (23) but was significantly associated with the malignization of leukoplakia, especially in relation to tobacco (but not alcohol) use (24). A correlation was also found between p16 methylation and higher-grade dysplasia (18,23,25,26). However, another study of patients in whom p16 methylation was detected reported that the time interval between methylation and OSCC onset varied widely (0-70 months, mean of 18 months), suggesting that p16 methylation may not be correlated with the pathological status of the lesion (27). On the other hand, a higher level (up to 4-fold) of methylation was found in histologically healthy samples from patients previously treated for OSCC, indicating a greater predisposition to tumour recurrence (24). Studies of saliva samples from patients with head and neck cancer (16,18,19) and with leukoplakia (25) reported 31.0-87.5% methylation of p16 in serum DNA. These levels, which are higher than those obtained in tumours, may be explained by the up to 4-fold higher concentrations of serum DNA in cancer patients versus healthy individuals (14). Serum analysis therefore represents an effective methylation detection method that is non-invasive, unlike biopsies. However, these results were obtained in studies of cancer patients, except for one (25) in patients with pre-cancer (34 patients with leukoplakia) that also found significant levels in saliva. Finally, studies of hair follicles (24,25) were able to establish that p16 methylation was related to the localization of leukoplakia onset. As in OSCC, p16 methylation was also studied in mucoepidermoid carcinoma of salivary glands (22), finding that the methylation (34.3%) was not significantly related to the pathogenicity of this cancer type, unlike the homozygous deletion of this gene.

**MGMT (O′-methylguanine-DNA methyltransferase).** This DNA repair gene has been observed to be inactive in cancer. As in the case of p16, MGMT has been widely studied in different types of sample from OSCC and from head and neck cancer in general. Studies have detected MGMT methylation percentages of 23 to 56.4% in tumour tissue (16,18,19,21,23,28) versus the 9% found in oral mucosa of healthy individuals (23). The data for salivary gland cancer are less clear: in the tumour, MGMT methylation of <20% was observed in 5 cell lines and 69 human salivary gland carcinoma samples, while methylation of up to 15% was found in healthy mucosa (29). However, reports of MGMT methylation of 68-73% in healthy perilesional tissue (21,28) and of 56 and 80% in leukoplakia samples (23,25,28) suggest that, as with p16, MGMT methylation may be related to an early stage of the lesion and might be useful as a prognostic or diagnostic factor. In fact, it was observed in lesions with a low-grade dysplasia and even in clinically normal oesophageal mucosa with no detectable histological lesion (23). Studies of saliva samples (16,18,25,19) obtained methylation percentages of 48-62%, indicating that saliva analysis is an effective method to detect methylation of this gene. Inhibition of MGMT was found to be related to an advanced stage of the disease, lymph node metastasis, a poor prognosis, tobacco use, and to increased genetic instability in pre-cancerous oral lesions (28).

**DAP-kinase (death-associated protein kinase).** This tumour-suppressor gene is implicated in apoptosis and potentially inhibits the appearance of metastases. For this reason, it has not been studied in pre-cancer or as a diagnostic factor. Methylation percentages of 18-27% have been reported in tumour tissue (7,16,18), which are not very elevated levels, although a high correlation was found with the presence of lymph node metastases (7,12,16,18). Findings on its presence in saliva samples from patients with head and neck cancer have ranged from 18 to 60%.

**RARβ2 (retinoic acid receptor).** RAR-β2 is the most important tumour suppressor gene of the RARβ family. Alterations in the gene that encodes the expression of this receptor have been found in various types of cancer and premalignant lesions. Suppression of RAR-β1 function due to methylation was observed in 67% of head and neck cancer samples >50% of head and neck tumour cell lines and pre-cancerous lesions, more frequently in the former, suggesting that methylation may increase during carcinogenesis (30). Furthermore, the prevalence was reported to increase with a higher grade of dysplasia (23). Olazs et al (31), among others, analyzed the effects of hypermethylation and loss of heterogeneity in genes that transcribe RARβ2 receptors, studying their role in retinoid therapy and chemoprevention. In their study of 89 primary head and neck tumours, methylation was the major cause of suppression of these receptors and was found in the tumour and as an early event in the carcinogenesis. RARβ2 methylation was not associated with tumour localization, stage or metastasis but was related to higher age, apparently due to the longer contact time between carcinogenic elements and mucosa (31). It also appears to be significantly related to tumours with an aggressive phenotype and to a poor survival prognosis (29).
E-cadherin. This transmembrane glycoprotein is involved in cell adhesion and has been related to the prognosis in invasive OSCC. Reports on the percentage methylation of the gene that encodes this protein range from 7 to 46% in patients with oral cancer (7,12,19) and show a >60% correlation with saliva samples (19). Its methylation is infrequent in salivary gland cancer (29). One study (32) determined the methylation of E-cadherin in seven cell lines of oral carcinoma, one of epidermoid cancer and one of malignant melanoma, and analyzed its association with the survival of motor neuron protein interacting protein 1 (SIP1). Previous studies had found that SIP1 was closely related to the loss of E-cadherin expression and to the prognosis of cancer patients. However, the above study found that methylation of this gene was highly involved in the inhibition of E-cadherin in cancer cells, which usually express SIP1 at multiple levels, suggesting that the methylation blocked expression of the gene independently of the proteins. Finally, it was reported (33) that alterations in pre-cancerous lesions may include E-cadherin methylation, which was observed in 85.4% of OSCC samples and in perilesional healthy mucosa samples from 33.3% of the cases, with 12.5% of these showing the same degree of methylation as the cancerous lesion.

Other genes. Other tumour suppressor genes have been investigated in the past few years, including PRTFDC1, whose alteration is usually due to deletion or mutation. However, its inhibition in OSCC lesions suggests that it may also be affected by epigenetic processes. In a recent study, methylation of this gene was detected in 17% of OSCC lesions and was not found to be related to tobacco or alcohol use, gender, age or tumour stage (34).

RAS association family 1 gene (isoform RASSF1A), located at 3p21.3, has also been widely studied in this context, showing 7.5% methylation in head and neck cancer (7) and 35% methylation in salivary gland cancer (29). It has also been associated with aggressive tumours and shorter survival time (29).

Methylation of p14 and p15 genes has been studied in the setting of OSCC. In the case of p14, methylation was found in 12% of a group of patients with a history of the disease, suggesting its association with a later stage in tumour formation (25). Methylation of p15 was observed in 29% of OSCC patients (19).

Researchers are currently investigating the methylation of new genes, including PTEN, a tumour suppressor gene involved in the inhibition of migration and tissue invasion, and RUNX3 (Runt-related transcription factor 3), an important component of the TNF-β-induced tumour suppression pathway. PTEN was analyzed in 113 tumours and the corresponding healthy perilesional tissue samples, finding a 71.8% reduction in gene expression due to methylation. It was previously demonstrated that this gene is inactivated by mutations or transcription losses and that the silencing of this gene is probably related to tumour genesis in OSCC (35). RUNX3 has been related to neurogenesis and thymopoiesis, and a decreased RUNX3 expression implies a reduced sensitivity of the growth inhibition effect and a reduced TNF-β-induced apoptotic activity. One of the mechanisms by which this protein is reduced is by methylation of the Cpg island and its gene expression. A study of 10 samples of healthy mucosa and 30 samples of OSCC with corresponding healthy perilesional mucosa detected methylation in 70% of OSCC samples and 53.3% of healthy adjacent mucosa samples, a non-significant difference. Methylation of this gene was not detected in any of the healthy mucosa control samples (36). There have also been recent studies on the methylation of other genes such as hMLH1 and hMSH2, which both participate in the DNA repair system (mismatch repair), concluding that their methylation may play a role in carcinogenesis and may be correlated with a tendency to develop multiple malignant lesions in the oral cavity (37).

4. Sampling for methylation study

Tissue samples. Samples are usually taken from pre-cancerous lesions, tumour lesions and/or healthy peritumoural tissue. Some authors use formalin-fixed and paraffin-embedded tissue for pathology and immunohistochemistry studies, cutting it into 5-µm sections, which are kept in gelatin capsules for haematoxylin-eosin staining (26,28,36). Other authors freeze samples at -80°C and cut them with a microtome (16,18-22,27,31,38). Others have used both procedures in the same study, freezing tissue samples of normal mucosa, mild dysplasia, severe dysplasia and OSCC at -70°C and cutting them into 5-µm sections (haematoxylin-eosin staining) and fixing samples from healthy patients with alcohol and embedding them in paraffin (23).

Saliva samples. After a 1-min mouthwash with 10 ml of distilled water, the patient expectorates into a sterile plastic tube that is then despatched to the laboratory. Samples are centrifuged at 2,500 rpm for 15 min and washed with phosphate-buffered saline (18,25). Supernatants are decanted and cell residues are frozen at -80°C in the same tubes (16,20).

Some authors took a few hair roots from each patient and stored them at -20°C to compare DNA of extraoral cells and examine the relationship of methylation with oral localization (24,25).

Formaldehyde-fixed and paraffin-embedded tissues are a very good source for genetic analysis. The handling, fixation and post-extraction care of the tissues are highly important. Tissues are washed with ethanol to remove paraffin. However, formaldehyde fixation may affect DNA acids by increasing the pH of the tissue, preventing its use for genetic analysis (39). The genetic material from paraffin-embedded tissues fixed in formaldehyde for >48 h is impaired (40).

The most effective method for DNA extraction from samples appears to be protease K digestion at 56°C for 3 h followed by denaturalization and treatment with phenol/chloroform and alcohol precipitate (19,20,25,40).

Methylation analysis technique is conducted with a methylation-specific PCR (MSP) described by Herman et al; it requires DNA treatment with sodium bisulphite to differentiate methylated and non-methylated bases (41). Methylation can also be used (42); it quantitatively analyses methylation using a fluorescence-based PCR method that allows various genes to be studied in several patients within
2 h (19). A novel technique known as methylation enrichment pyrosequencing (MEP) has been developed that overcomes
the shortcomings of MSP, which tends to yield false positive
results and is not reliable in samples with a low concentration
of DNA and high number of cycles. Pyrosequencing avoids
false positives; the treatment is controlled with bi-sulphate,
and it has a high sensitivity and capacity to detect methylation
in samples with a low DNA content (27).

5. Relationship with other tumours

Methylation of p16, among other genes, has also been
detected in 24% of hepatic carcinomas, and the lack of
its expression has been associated with tumours with high-
versus low-grade malignancy; therefore, it appears to be
related to the malignant phenotype of this liver tumour
(38). Methylation of DAP-kinase has been related to tumour
spread and to lung cancer, leukaemia and lymphomas
(16,17,43,44). MGMT is frequently inactivated in brain, lung
and colorectal cancers and lymphomas (44), and suppression
of E-cadherin is observed in invasive gastric carcinomas
and in those with high-grade dysplasia (32). Roth et al
reported that certain genes (p16, MGMT, DAP-kinase) were
methylated in >90% of a series of oesophageal carcinomas,
concluding that methylation was a frequent event in the
development of this cancer. They also found a tendency
for methylation to increase with higher grade of epithelial
dysplasia, with a more marked change between mild and
severe dysplasia (23).

6. Relationship with tobacco use

In oral cancer, the methylation of p16 and E-cadherin
appears to be related to the tobacco habit, since exposure to
tobacco directly or indirectly induces damage to the CpG
islands in certain genes, eliminating protective mechanisms
or inducing DNA-methyltransferase activity. Hence, tobacco
plays a major role in the methylation of these genes, and
an earlier start and greater intensity (cigarettes/year) of the
habit increase the likelihood of aberrant methylation of
p16 and E-cadherin, respectively, in head and neck cancer.
In contrast, no relationship has been found between gene
methylation and alcohol use (7).

Methylation of p16 has also been detected in 1-15% of
oral mucosa samples from healthy smokers with no history
of pre-cancerous lesions (18,45).

Methylation of p14 is especially frequent in pre-cancerous
lesions associated with betel nut chewing and may
reduce the normal transcription of p53 (tumour suppressor
gene associated with p14). However, p16 methylation
did not differ between betel chewers and non-chewers but did
differ between tobacco smokers and non-smokers (24).

7. Relationship with progression and malignization

MGMT has been detected in peritumoural oral mucosa and
in epithelial hyperplasia and dysplasia, with a higher percentage
methylation in the latter, suggesting that its methylation is an
early event in oral carcinogenesis (28). Healthy mucosa of
OSCC patients also presents methylation of MGMT and p16,
which may therefore serve as disease markers. Methylation
can be considered an event at the beginning of carcinogenesis
that persists over time and gives rise to cell division, increasing
the capacity for tumour recurrence in patients already treated
for a primary tumour (21,24,25).

It has also been reported that the methylation of genes
encoding proteins that control intercellular binding and
apoptosis (e.g., DAP-kinase, E-Cadherin, and CLDN3) may
promote invasive tumours and increase the likelihood of
metastasis (16,17).

8. Reversibility of methylation

In contrast to genetic changes, changes at epigenetic level
are potentially reversible. Consequently, methylation and
acetylation inhibition could be studied as anti-cancer therapy.
A potent specific inhibitor of methylation is 5-aza-20-deoxy-
cytidine, which reverses methylation in vitro and has been
used in the treatment of leukaemias and myelodysplasias.
However, this inhibitor can also activate other genes that are
silenced under normal conditions, resulting in new alterations
that may not be beneficial for the patient. Nonetheless, there
is interest in the fact that methylation blocks genes that confer
chemoresistance to certain pharmaceuticals; for instance,
protein caspase-8 reactivates with 5-aza-20-deoxycytidine
and restores chemotheraputic sensitivity (7,11,12,30,32). It has
also been reported that retinooids may revert the carcino-
genesis process by numerous mechanisms, including the
modulation of epithelial differentiation and proliferation by
gene expression regulation. One of their receptors is RARβ2,
which is suppressed in early stages of head and neck cancer
and whose restoration is associated with a clinical response to
cancer (31).

9. Conclusion

Knowledge of the hypermethylation of certain genes may
contribute to a deeper understanding of cancer. Hyper-
methylation is found in tumour lesions, cancer precursor
lesions and in healthy mucosa, but in different percentages,
so it has been proposed as a diagnostic and prognostic
molecular marker to assed a higher risk of malignant lesion
development. The genes that appear to be most implicated in
malignant oral lesions are p16, MGMT, RARβ2, E-cadherin
and DAP-kinase based on this review. P16 and RARβ2 are
tumour suppressor genes involved in the cell cycle so the
silencing of these genes would imply an impaired cell
reproduction, MGMT is a DNA repair gene which is impor-
tant to preserve the DNA integrity, and the last two genes
participate mostly in the progression of OSCC to metastasis,
as being the E-cadherin a cell-to-cell adhesion controller
and DAP-kinase an apoptosis controller. However, further
studies are necessary to verify the true usefulness of this
process in the early diagnosis of pre-cancerous lesions and
to determine them as related prognostic factors.

References

1. Oliver RJ, Dearing J and Hindle I: Oral cancer in young adults:
report of three cases and review of the literature. Br Dent J 188:


