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Epigenetic Profiling of Oral Cancer

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1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of oral neoplasm, accounting for over 90% of all mouth malignancies and 38% of head and neck tumors. Worldwide, OSCC is the eighth most common human cancer, with more than 500,000 new cases being diagnosed every year. Surprisingly, the number of annual deaths for this disease has practically not changed in the last 30 years (Funk, G.F.; Karnell, L.H., 2002).

This is because invasive surgical treatments (involving both oral cavity and neck) are still the only effective way to treat OSCC; thus detection in early stages could dramatically improve cure rates and quality of life by minimizing invasive surgery (Scully, C., 1995). To improve diagnosis and prevention, researchers have spent considerable effort to understand which genetic and/or environmental changes may be related to this tumor. Although environmental risk factors associated with development of oral cancer have been sufficiently understood (smoking, alcohol, betel, diet, living habits, etc.), knowledge of the genetic bases in oral carcinogenesis is still a challenging task. OSCC is a result of multiple genes alterations, which are modulated by individual predisposing conditions and environmental influences. Furthermore, in the last ten years a new category of non-genetic events able to modify gene expression has been massively investigated: the so called 'epigenetic phenomena' (Bird, A., 2007).

Epigenetic factors are non-genetic phenomena which interfere with genes expression. Such modifications pass on successive generations of cells, even if there is no mutation in corresponding genes. Epigenetic events are linked with carcinogenesis when one or more

oncogenes/tumour suppressors are directly or indirectly affected such that their expression and function may be permanently altered (Feinberg, A.P., 2001)

Cellular aging, risk factors and, as recently discovered, chronic inflammation via mediators, such as IL-6, may be potential inducers of epigenetic alterations in oral mucosa cells. It is a general belief that these alterations would accumulate in the normal-appearing mucosa while carcinogenesis is in progress, or before any tumor lesion is detected.

Three major types of such epigenetic mechanism are currently known: DNA hyper-methylation, histone code changes and RNA interference.

1.1 DNA hyper-methylation

Methylation is the biochemical addition of a methyl group(-CH₃) to a molecule. In cellular biology, this refers to methylation of DNA, RNA and proteins. Protein methylation has been extensively studied recently and it is an essential post-translational modification that affects its function. RNA methylation is less understood and it probably plays a role in message stability. DNA methylation is the only normally occurring modification of DNA from bacteria to humans, although it plays a different role in eukaryotes and in prokaryotes (Baylin, S.B.; Herman, J.G., 2000)

In mammals, methylation physiologically affects cytosine bases incorporated into DNA, primarily when it is followed by a guanosine (hence it is defined as Cytosine-phospho-Guanosine or CpG methylation). CpG sites are distributed unevenly in the genome. They are rare in 99% of the human genome, and most of these CpG sites are modified by methylation. It follows that about 1% of the genome consists of CpG rich areas, typically 500-2000 base pairs long, and are named CpG islands. About half of all CpG islands corresponds to transcription start sites and promoters of expressed genes, while non-promoter associated CpG islands are less well understood and can be methylated in normal tissues. About half of all genes has CpG islands in their promoters. Most promoter-associated CpG islands are free of methylation, regardless of the expression state of the associated gene. Genes that do not have CpG islands in their promoters show different patterns of methylation; some rare CpG sites are typically methylated in their transcription start areas when the gene is inactive, and un-methylated when the gene is active, but non-CpG island methylation does not prevent gene expression; it can be reversed quickly upon gene activation and may serve primarily to regulate the degree of acute gene activation by transcription factors. DNA hyper-methylation in promoter-associated CpG islands is considered the same way as proper genetic modification for its ability to influence genes expression. A switch from un-methylated to methylated CpG islands was first demonstrated on the inactive X-chromosome in women in the rare instances when a cell needs mono-allelic expression for normal function, and then in about 100 genes that are imprinted (Mono-allelic expression based on parental origin). DNA hyper-methylation is now regarded as an epigenetic mechanism of gene silencing in mammals. The DNA-methyltransferase enzymes (DNMT1, DNMT3a and DNMT3b) are essential to establish and preserve normal patterns of DNA methylation, and are helped in this function by other proteins, such as DNMT3L (Robertson, K.D., 2001). Hyper-methylation role in oral carcinogenesis will be treated in the second section of this chapter.

1.2 Histone code changes

In eukaryotic cells, DNA is wrapped around an octameric histone core to form the nucleosome, the fundamental subunit of chromatin. Many residues in the histone proteins are subject to reversible post-translational modifications, emerging as important epigenetic mediators of gene expression changes. There are numerous possible modifications of histone tails which regulate genes expression, such as acetylation, methylation, ubiquitylation and phosphorylation. Histone methylation, mediated by histone methyltransferases (HMTs), can have either positive or negative effects on gene expression. Increase in histone acetylation generally correlates with gene activation, and results from the dynamic interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Furthermore, histone modifications play an important role in chromosome structure, and silencing marks are enriched at silenced loci, such as imprinted genes, suggesting that they play a role there as well. The ultimate mediators of histone methylation associated gene silencing appear to be proteins that bind specific modified histone and recruit effector protein complexes. Among these, the ones which seems to play a significant role in carcinogenesis belongs to ING protein family. The ING proteins (ING 1-5) are involved in cell cycle, apoptosis and senescence. The ING family emerged as putative tumor suppressor gene (TSG), and its major mechanism of activity entails the conserved plant homeodomain (PHD), which binds to histones in a methylation-sensitive pathway, e.g. binding histone H3 tri-methylated on lysine 4 (H3K4me3), suggesting that ING proteins may contribute to the maintenance of the epigenetic code. Furthermore, ING family members contain nuclear localization signals and N-terminal sequences, which play an important role in the interaction with histone acetyltransferase (HAT) and histone deacetyltransferase (HDAC). Although ING proteins have the same PHD motif, the variation in the N-terminal dictates the differences in the suppressive ability of ING in various tumors. ING proteins are involved in transcriptional regulation of genes, such as the p53-inducible gene p21. In cancer cells, INGs mRNA levels are often lost or suppressed but their genes are rarely mutated; indeed, the inactivation of the normal function is achieved through allelic loss of genomic regions containing the ING gene, alteration in the ING promoter region, variation of mRNA splicing efficacy or reduced mRNA stability. It is most probably a combination of these aberrant mechanisms that resulted in reduced levels of ING protein. Furthermore, the mechanism of suppression of ING expression may be related to the abnormally high methylation levels of the ING gene promoter, which have been related to low transcript levels. Recently, the potential roles of ING proteins as prognostic biomarkers, detector of aggressive behavior of tumors as well as predictive factor of chemo-radiotherapy response, have been hypothesized. Emerging evidence on the function of ING and related regulatory mechanisms strongly points to ING as a candidate TSG and therefore a potential target in the molecular therapy of some types of cancer (Gunduz, M.; Demircan, K., 2009; Gunduz, M.; Gunduz, E.; Rivera, R.S., 2008). Being research on INGs in its early stages, this topic will not be treated in this chapter.

1.3 RNA interference (RNAi)

In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNA interference in the nematode worm *C. elegans*, which they published in 1998 (Fire, A.; Xu, S., 1998).

RNA interference (RNAi) is a system involved in controlling gene activation in living cells. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are considered as key mechanisms related to RNA interference. The scientific community considers RNA interference the breakthrough biological discovery of the decade with the potential to change how diseases are treated. Being RNAi machinery in every cell and any gene a potential target, any disease caused by or greatly exacerbated by the expression of a dominant gene can in principle be treated by RNAi. This means that the list of potential indications is long. Diseases that are intractable or poorly responsive to current therapy include cancer, neurodegenerative disease, viral infection, and macular degeneration, and these are indeed the most studied disease models (Dykxhoorn, D.M.; Palliser, D., 2006). In this chapter we discuss the possible role of siRNA and miRNA in oral cancer.

- **Small interfering RNAs (siRNA)**, sometimes referred to as **short interfering RNAs** or **silencing RNAs**, represent a class of double-stranded RNA molecules, 20-25 nucleotides in length, that play a notable role in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene, but also in RNAi-related pathways as well as in antiviral mechanism or in shaping the chromatin structure of a genome. siRNAs were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England, as part of post-transcriptional gene silencing (PTGS) in plants. The group published their findings in *Science* in a paper titled "A species of small antisense RNA in post-transcriptional gene silencing in plants" (Hamilton, A.; Baulcombe, D., 1999). Shortly thereafter, in 2001, synthetic siRNAs were shown to be able to induce RNAi in mammalian cells by Thomas Tuschl, and colleagues in a paper published in *Nature* (Elbashir, S.; Harborth, J., 2001). This discovery led to a surge in interest in harnessing siRNA for biomedical research and drug development. It is expected that in some situations turning off or knocking down the activity of a gene with an siRNA could produce a therapeutic benefit. However, applying RNAi via siRNAs to living animals, especially humans, poses many challenges. Under experiments, siRNAs show different effectiveness in different cell types in a manner as yet poorly understood. Anyway, the effectiveness of gene silencing achieved with siRNA surpasses what has been possible in the past using other small nucleic acids, such as antisense oligonucleotides or ribozymes. In a head-to-head comparison, expression of siRNAs knocked down genes fold about 100-1000 times more efficiently than antisense oligonucleotides. The high efficiency may be related to the fact that one siRNA can be used over and over to guide the cleavage of many mRNAs. The high efficiency may also be due to protection of the active component of the siRNA (the antisense or guide strand) from digestion by endogenous RNases. Local siRNA administration has shown benefit in small animal models involving the lung, vagina, subcutaneous tissue, muscle, eye and central nervous system (Dykxhoorn D.M.; Palliser, D., 2006). Although siRNA are emerging as targeted cancer therapeutics inhibiting tumor-specific proteins or pathways, they have not totally eliminated the problem of toxicity. In fact, siRNA therapeutics are hindered by poor intracellular uptake, limited blood stability and non-specific immune stimulation. To address these problems, ligand-targeted, sterically stabilized nanoparticles have been adapted for siRNA. Intravenous administration into tumor-bearing mice gave selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor and

inhibition of both tumor angiogenesis and growth rate. The results suggest achievement of two levels of targeting: tumor tissue selective delivery via the nanoparticle ligand and gene pathway selectivity via the siRNA oligonucleotide. This opens the door for better targeted therapy with both tissue and gene selectivity. (Schiffelers, R.M.; Ansari A., 2004). Results of phase I studies of the first two therapeutic RNAi trials (indicated for age-related macular degeneration, aka AMD), reported at the end of 2005, indicated that siRNAs are well tolerated and have suitable pharmacokinetic properties (Tansey, B., 2006). Other trials have indicated that Ebola-targeted siRNAs may be effective as post-exposure prophylaxis in humans, with 100% of non-human primates surviving a lethal dose of Zaire Ebolavirus, the most lethal strain (Geisbert, T.W.; Lee, A.C.H., 2010). The emerging siRNAs role in oral carcinogenesis will be treated in the third section of this chapter.

- **Micro-RNAs (miRNAs)**, first identified in *Caenorhabditis elegans* in 1993, are small non-coding RNAs, which play an essential role in modifying genes expression. They are composed of 20–22 nucleotides, typically excised from 60–110 nucleotide foldback RNA precursor structures (Kim V.N. & Nam, J.W, 2006; Pasquinelli, A.E., Hunter, S., 2005). miRNAs seem to be implied in regulation of one third of the genes present in the human genome. Hundreds of miRNAs are expressed in eukaryotic cytoplasm, where they effect their action by mediation on RNA transcript cleavage and/or regulation of translation quote (Bentwich, I.; Avniel, A., 2005; Berezikov, E.; Guryev, V., 2005).

Since 2000, miRNAs have been investigated en mass and their mechanism of production and mode of action have been well characterized. The biogenesis of miRNAs involves a complex protein system, including members of the Argonaute family, Polymerase-II-dependent transcription and the RNase IIIs Drosha and Dicer (Lee, Y.; Ahn, C., 2003; Bartel, D.P., 2004). In particular, miRNAs are transcribed by RNA polymerase II or RNA polymerase III as a part of an intron of mRNA or as an independent gene unit; then, initially transcribed miRNAs, which can be several hundred to thousands of nucleotides long, are cleaved into a <100 nucleotide stem-loop structure by a type III RNase, named Drosha (Bernstein, E.; Caudy, A.A., 2001).

These pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (Kim, V.N., 2004), and once there, the pre-miRNAs undergo another round of cleavage by Dicer, another type III RNase. The fully cleavage process results in miRNAs approximately 18 to 24 nucleotides long. These mature miRNAs- which are usually named “miR X”, being x a cardinal number - are bound by a protein complex, called RNA-induced silencing complex (RISC). This active miRNA-RISC complex binds to target mRNAs based on sequence homology between the miRNA and the mRNA. Typically, the miRNA blocks mRNA translation and/or leads to mRNA degradation. Because miRNAs bind with imperfect complementary to target mRNAs, it is estimated that one miRNA is capable of binding >100 different mRNAs with different binding efficiencies. The number of human miRNAs is in excess of 450, twice as many as initial calculations indicated and more than 1,000 predicted miRNA genes are awaiting experimental confirmation. With about 1,000 miRNAs expected to be present in the human genome, it is postulated that ~30% of all mRNAs are post-transcriptionally regulated by miRNAs. Hence they are considered to play important roles in cell growth, differentiation, apoptosis, stress response, immune response, and glucose secretion. It has recently been proved that miRNAs are differentially expressed in cancer

cells compared with normal cells, and it seems that miRNAs cluster (changing their number in the order of ten to hundreds) can characterize different types of solid and haematopoietic tumor cells more accurately than mRNA, suggesting that there is a link between miRNAs and cancer and that miRNAs can be used to detect cancerous/precancerous condition. miRNAs located in genomic regions amplified in cancers (such as the *miR-17-92* cluster) function as oncogenes, whereas miRNAs located in portions of chromosomes deleted in cancers (such as the *miR-15a-miR-16-1* cluster) function as tumour suppressors (Kent, O.A. & Mendell, J.T., 2006; Gregory, R.I. & Shiekhattar, R., 2005). The genomic abnormalities found to influence the activity of miRNAs are represented by chromosomal rearrangements, genomic amplifications or deletions and mutation and also by epigenetic silencing (Kozaki, K.; Imoto, I., 2008).

Abnormal expression of mature miRNAs or of their precursor have important consequences for the expression of various protein-coding genes involved in malignant transformation if compared with the corresponding normal tissues, and can be found by various genome-wide techniques (including different microarray platforms or bead-based flow cytometry) (Calin, G. A. *et al.*, 2004). It has been stated that miRNA expression fingerprints correlate with clinical and biological characteristics of tumors, including tissue type, tumor origin, differentiation, aggression and response to therapy (Lu, J. *et al.*, 2005). Volinia, S. *et al.*, 2006). Germline sequence abnormalities were identified in microRNA (miRNA) genes or transcripts, and in targeted sequences in messenger RNAs (mRNAs) that interact with miRNAs. This fact seems to partially explain familial predisposition to cancer (He, H. *et al.*, 2005). Finally, miRNAs should efficaciously affect and improve cancer diagnosis and prognosis types. The miRNAs role in oral carcinogenesis will be treated in the third section of this chapter.

2. Gene promoters hyper-methylation

Hyper-methylation of cytosine base in CpG islands of gene promoters is an epigenetic phenomenon able to down regulate the expression of genes. When an oncogene expression is influenced, this phenomenon is directly linked with carcinogenesis (Kulkarni, V. & Saranath, D., 2004).

In oral district, many genes are considered to cause OSCC if their methylation status is altered. Among the considerably high amount of investigated genes, only the significant ones in oral cancer will be considered.

2.1 Inhibitors of canonical WNT-pathway

WNT proteins are a large family of secreted glycoproteins activating at least three signalling pathways: the canonical WNT-pathway or WNT- β -catenin, the non-canonical WNT pathway or planar cell polarity (PCP) and WNT-Ca²⁺ pathway (Cadigan, K.M. & Nusse, R., 1997).

The canonical pathway operates by stabilizing β -catenin. The stabilization of β -catenin in the canonical pathway translates a WNT signal into the transient transcription of T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors to stimulate the expression of target genes; the process results in initiating cellular proliferation. The

main receptor of secreted WNT proteins at plasma-membrane is the protein Frizzled (Fz), but other Fz co-receptors are required for proper WNT signalling, such as low-density lipoprotein receptor-related proteins (LRP-5 or LRP-6). WNT-inhibitors may be classified into two types: a) the ones that interfere with WNT activity by binding to LRP-5 and LRP-6, including Dickkopf (DKK) proteins, and b) the ones that interact directly with WNT proteins, including secreted Frizzled related proteins (SFRPs) and WNT inhibitory factor -1 (WIF-1). DKK proteins interact with the co-receptors LRP-5/6 and inhibit signalling by disrupting the binding of LRP-6 to the WNT/Fz ligand-receptor complex (Aguilera, O.; Muñoz, A., 2007).

SFRPs, a family of highly conserved glycoproteins, share structural similarities with the Frizzled receptor family of proteins and antagonize the WNT pathway at the level of receptor-ligand binding (Bovolenta, P.; Rodriguez, J., 2006; Rattner, A.; Hsieh, J.C., 1997). Thus, they play a crucial role in cell proliferation and differentiation, epithelial-mesenchymal communication and embryogenesis (Bafico, A.; Gazit, A., 1999).

WIF-1 is a secreted inhibitor of WNT signalling and its expression results in cell growth inhibition via G1 (Tang, Y.; Simoneau, A.R., 2009). Mechanisms of WIF-1-induced G1 arrest include (a) SKP2 (SKP2 gene contains two TCF/LEF-1 consensus binding sites within the promoter) down-regulation leading to p27/Kip-1 accumulation and (b) c-myc down-regulation releasing p21/WAF-1 transcription. Chronic activation of WNT can be caused by loss of WNT inhibitors through epigenetic silencing. Although dysregulation of the WNT- β -catenin pathway is a frequent event in several human cancers (Clevers, H., 2006; Giles, R.H.; van Es, J.H., 2003), its potential implications in oral cancer has been investigated in only a few works. SFRP1 promoter is hypermethylated in 24% cases of OSCC according to Sogabe et al. (Sogabe, Y.; Suzuki, H., 2008), while Pannone et al. found it was statistically significant less methylated in OSCC than in healthy mucosa; according to Pannone et al., other genes as SFRP2, 4, 5, WIF1 and DKK2 are statistically significant hyper-methylated in OSCC if compared to healthy mucosa (Pannone, G.; Bufo, P., 2010). Sogabe et al. (Sogabe, Y.; Suzuki, H., 2008) also found that SFRP2 and 5 were hyper-methylated in 36% and 16% of tumoral cases respectively.

2.2 Tumor-suppressor genes

A tumor suppressor gene is a gene encoding for a protein which functions include avoiding atypical transformation of cell population, preventing cancer development, protecting a cell from the inauspicious path to cancer. Mechanisms by which a tumor suppressor protein effects its function can be very different: controlling cell cycle genes - including oncogenes -, controlling/repairing mismatches in DNAs duplication, initiate apoptosis in case of cellular atypia, allowing cell adhesion to prevent tumor cells dissociation. Inactivation of several tumor-suppressor genes has been attributed to aberrant hyper-methylation of their promoter regions. E-cadherin, p16, p15, hMLH1, MGMT and many others are well-known tumor-suppressor genes, that are considered to be widely inactivated by methylation in cancers (Muthusamy, V.; Nobuo, T., 2003).

E-cadherin, a member of the cadherin superfamily, is a calcium-dependent homotypic epithelial cell-cell adhesion glycoprotein (Figure 1). E-cadherin is located on the surface of normal epithelial cells and decrease of E-cadherin expression has been found in cancers. It

has been postulated that the loss of this protein, facilitates tumor cell dissociation and metastasis. Diminished E-cadherin expression has been documented in association with the acquisition of invasiveness in vitro and poor prognosis in many carcinomas. E-cadherin promoter hyper-methylation, in oral squamous cell carcinomas, is one of the most investigated phenomenon (Chen, Q.; Lipkina, G., 2004). In OSCC epigenetic hyper-methylation occurs in 35-85% cases; only in very rare cases the difference of E-cadherin promoter hyper-methylation between carcinoma and healthy mucosa is not statistically significant. Supic et al. found that hyper-methylated E-cadherin OSCC patients had a worse survival ($p=0.039$), and according to Chang et al. recurrent OSCCs compared to primary tumors are more hyper-methylated and the difference is statistically significant (Chang, H.W.; Chow, V.; 2002).

Yet, it has been found by de Moraes et al. and Yeh et al. that promoter hyper-methylation is not related to E-cadherin expression (according to de Moraes et al. in some cases this ratio may be even inverse) (de Moraes, R.V.; Oliveira, D.T., 2008; Yeh, K.T.; Shih, M.C., 2002).

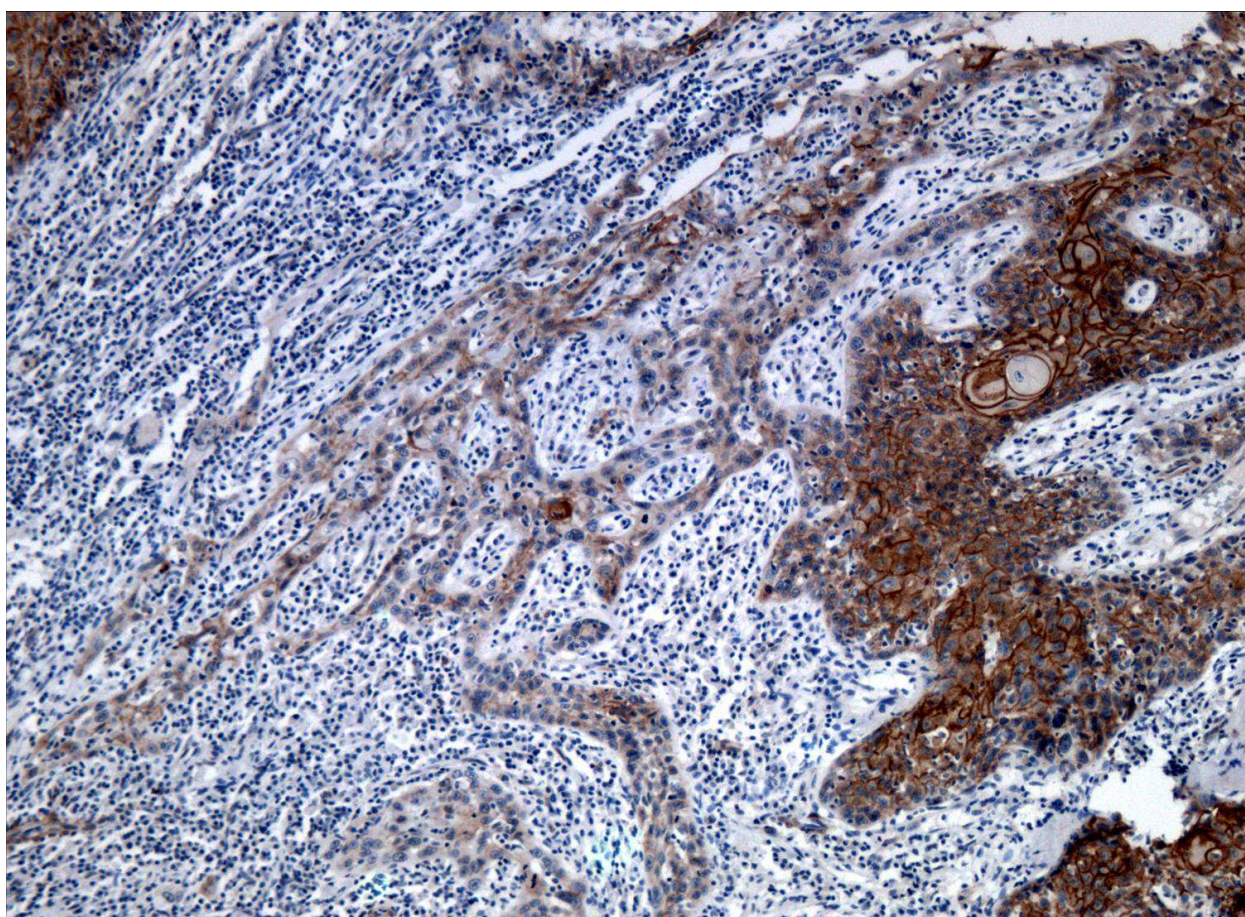


Fig. 1. Immunohistochemical expression of E-cadherin in oral cancer. (LSAB-HRP-, nuclear counterstaining with haematoxylin, original magnification x100). In this representative case of moderately differentiated oral cancer, superficial neoplastic cells keep membranous protein expression. In the deeply infiltrating neoplastic cells E-cadherin loses membranous staining and assumes cytoplasmic positivities.

p16 is a cycline-dependent kinase (CDKN2A) inhibitor involved in regulation of the cell cycle by cyclin D-Rb pathway, which control is virtually lost in all tumors. This tumor suppressor protein is one of the INK4 class members of cell-cycle inhibitors. The expression of p16 retains Rb-family proteins in a hypo-phosphorylated state, which promotes the binding of E2F to achieve G1 cell-cycle arrest. In OSCC, p16 promoter is hyper-methylated in 23-86,8% cases (Huang, M.J.; Yeh, K.T., 2002; Liu, H.W.; Hu, B.Q., 2005; Merlo, A.; Herman, J.G., 1995). Moreover, Hall et al. found that 57% of pre-malignant lesions with hyper-methylated p16 underwent malignant transformation, while non-p16-hypermethylated pre-malignant lesions evolved into cancer in 1% cases (Hall, G.L.; Shaw, R.J., 2008).

When a difference between metastatic-non metastatic OSCC was investigated, p16 promoter was more hyper-methylated in metastatic OSCCs than in non-metastatic ones and the difference was statistically significant. Dong et al. found an high correlation between promoter hyper-methylation and un-expression of p16 (Dong, Y.Y.; Wang, J., 2006).

Adjacent to p16 gene lies the p15 gene, also called CDKN2B gene. Takeshima et al. reported that its promoter is never methylated in healthy epithelial cells, while it shows a certain degree of hyper-methylation in OSCC (Takeshima, M.; Saitoh, M., 2008), also confirmed by Viswanathan et al. (Viswanathan, M.; Tsuchida, N., 2003).

DNA mismatch repair (MMR) is a system for recognizing and repairing mistakes, arisen during DNA replication and recombination, as well as repairing some forms of DNA damage. It is apparent how bad function/low expression of proteins involved in these mechanisms is strictly related to cancer. Among all the genes involved in this system, hMLH1 and hMSH2 promoter hyper-methylation have been investigated to understand their potential role in oral carcinogenesis (González-Ramírez, I.; Ramírez-Amador, V., 2010). hMLH1, the human homolog of bacterial Mut L, is involved in mismatch repair (Veigl, M.L.; Kasturi, L., 1998). hMLH1 promoter is hyper-methylated in 0-76% oral squamous cell carcinoma cases. When protein expression was investigated, it was found unexpressed in 32-36% only among hyper-methylated cases. Czerninski et al. reported that in their experience 100% of patients with multiple oral malignancies showed hyper-methylation in hMLH1 or hMSH2 compared with 31.5% of single tumor patients, although often hyper-methylated cells expressed these 2 proteins anyway. They concluded that hMLH1 and hMSH2 might be related with predisposition to develop multiple oral malignancies (Czerninski, R.; Krichevsky, S., 2009).

DAPK Death-associated protein kinase (DAPK) is a calmodulin-regulated serine/threonine kinase and possesses apoptotic and tumor-suppressive functions. Although it is unclear whether DAPK elicits apoptosis-independent activity to suppress tumor progression, it has been hypothesized that it may affect its apoptotic function to suppress tumor progression by regulating cell polarity during migration. Supic et al. reported that DAPK promoter methylation is higher in OSCC mucosa than in healthy control in a statistically significant way, and that its presence on surgical margins is an independent prognostic factor for overall survival (Supić, G.; Kozomara, R., 2009). Promoter hyper-methylation of DAPK gene detected in surgical margins may be a useful molecular marker to explain the poor survival of some OSCC patients.

MGM is the gene involved in repairing methylated guanosine residues due to alkylated carcinogens. Alkylating agents are known to be carcinogenic because of the formation of O6-alkylguanine from alkylation of the O6 position of DNA and they are responsible for malignant transformation and mutations. O6-methylguanine-DNA methyltransferase (MGMT) plays a role in the mechanism of resistance to alkylating agents by repairing O6-alkylguanine by removing the alkyl group and restoring guanine (Rosas, S.L.; Koch, W., 2001). Aberrant promoter hyper-methylation of MGMT in OSCC has been found in 12,2-73,7% cases and widely considered related to oral carcinogenesis (Kato, K.; Hara, A., 2006; Kordi-Tamandani, D.M.; Moazeni-Roodi, A.K., 2010; Taioli, E.; Ragin, C., 2009).

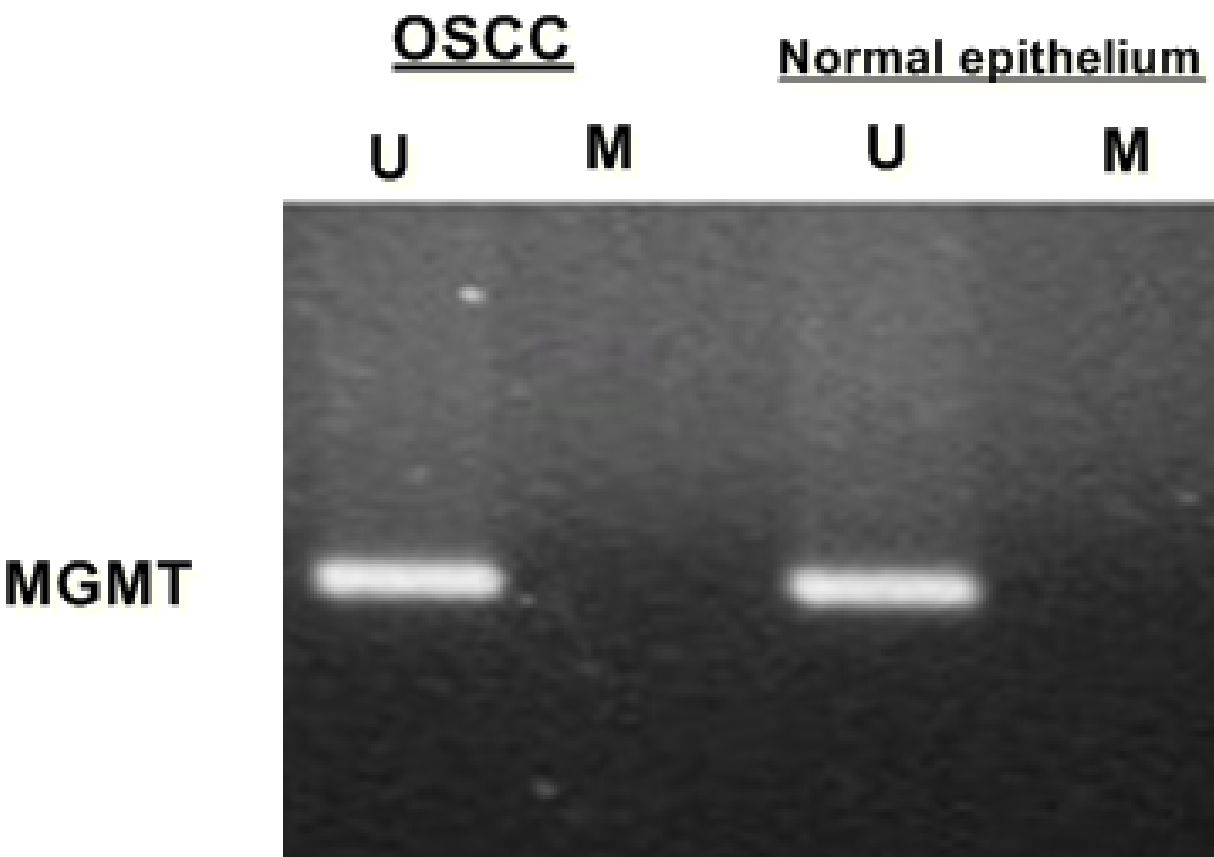


Fig. 2. Promoter methylation status of MGMT in OSCC.
In our study MGMT represents a frequently un-methylated gene. M, Methylated DNA; U, un-methylated DNA. Methylation specific (MSP) PCR technique, performed on formalin-fixed, paraffin-embedded tumor and control samples.
To test the integrity of isolated DNA the wide housekeeping haemoglobin gene was amplified by PCR and visualized by gel electrophoresis for both control and pathological samples. The DNA quantity was evaluated by a Nano Drop Spectrophotometer (CELBIO). Sodium bisulfite modification of 100µg DNA for each sample was also performed. All methylation-specific PCRs were optimized to detect >5% methylated substrate in each sample. Each experiment was performed in triplicate.

RUNX3 (Runt-related transcription factor 3) is a protein member of the runt domain-containing family of transcription factors. This protein interacts with a high amount of enhancers and promoters, either activating or suppressing transcription, and with other transcription factors. It functions as a tumor suppressor, and, in some cancers, the gene is frequently deleted or transcriptionally silenced. Gao et al. reported that RUNX3 gene and protein were under-expressed in OSCCs due to promoter hyper-methylation, with frequent protein delocalization. The study showed how both down-regulation of protein expression and protein mislocalization were correlated with the differentiation grades in OSCCs. They consider RUNX3 a useful diagnostic marker and a potential therapeutic target for OSCC, playing an important role in oral carcinogenesis (Gao, F.; Huang, C., 2009). On the other hand, de Freitas Cordeiro-Silva et al. reported a not-statistically-significant hyper-methylation of RUNX3 gene in oral neoplastic mucosa (de Freitas Cordeiro-Silva, M.; Oliveira, Z.F., 2011).

Other tumor suppressor genes that have been found significantly hyper-methylated in oral cancer are: Deleted in Colon Cancer (DCC, encoding a transmembrane protein with structural similarity to neural cell adhesion molecule), Ras association domain-containing protein 1 (RASSF1, a protein similar to RAS effector protein) (Tran, T.N.; Liu, Y., 2005), Kinesin-like protein 1A (KIF1A, a member of kinesines superfamily), Nidogen-2 and Homeobox protein Hox-A9 (NID2 and HOXA9, involved in cellular differentiation and apoptosis) (Guerrero-Preston, R.; Soudry, E., 2011), Endothelin receptor type B (EDNRB, a member of endothelin receptors family).

2.3 Retinoids

Retinoids, analogues of retinol (vitamin A) have been widely tried in the prevention of oral squamous cell cancer, and as a cure for its precancerous lesions. In pre-clinical studies, retinoids have been shown to suppress carcinogenesis in a variety of epithelial tissues, including skin, oral mucosa, trachea, prostate, lung, bladder and mammary glands (Shao, Z.; Shen, Z., 1995; Lotan, Y.; Xu, X.C., 2000; Xu, X.C.; Sozzi, G., 1997). Many of the effects of retinoids result from modulation of gene expression by at least 2 distinct classes of nuclear receptor: retinoic acid receptors (RAR-a, b and g) and retinoid X receptors (RXR-a, b and g), belonging to the steroid/thyroid hormone superfamily, even if the mechanisms underlying these clinically significant anti-carcinogenic activities are not completely understood. Defects in retinoid receptor structure, expression and function have been detected in various types of cancer cell *in vitro* and *in vivo* (Gebert, J.F.; Moghal, N., 1991; Geisen, C.; Denk, C., 1997).

These defects may enhance cancer development by interfering with retinoid signaling, thereby abrogating the putative physiological anti-carcinogenic effects of natural retinoids (Lotan, R., 1996). Reduction in RARb mRNA has been observed in several malignant tumors (Caliaro, M.J.; Marmouget, C., 1994; Rochaix, P.; Monteil-Onteniente, S., 1998) and in oral malignant-premalignant lesions (Chakravarti, N.; Mathur, M., 2001; Xu, X.C.; Ro, J.Y., 1994). Rar-beta 2 promoter hyper-methylation was investigated by Youssef et al. and found in 66% of OSCC cases ($p=0,002$ if compared to healthy mucosa) (Youssef, E.M.; Lotan, D., 2004).

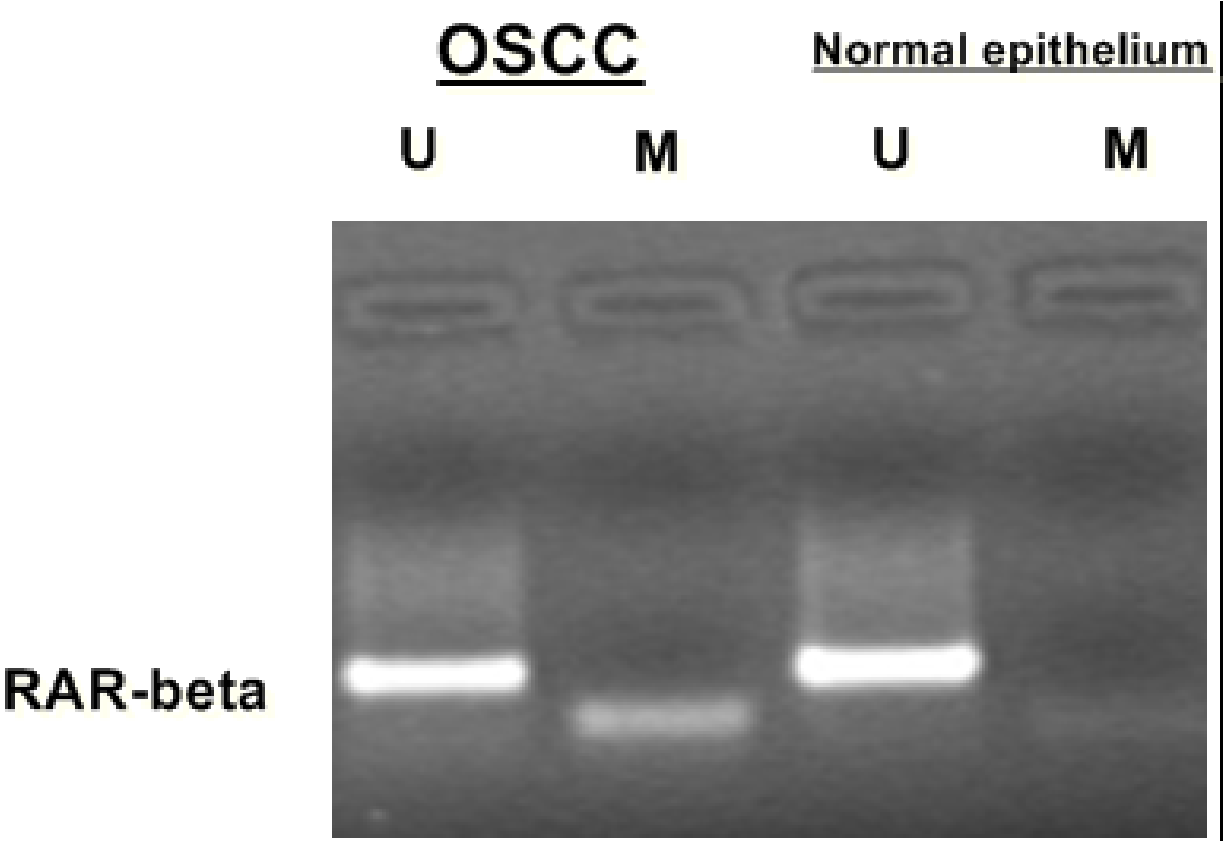


Fig. 3. Promoter methylation status of RAR-beta in OSCC.
A representative case of OSCC with methylated promoter of RAR-beta. M, Methylated DNA; U, un-methylated DNA. Methylation specific (MSP) PCR technique, performed on formalin-fixed, paraffin-embedded tumor and control samples.
To test the integrity of isolated DNA the wide housekeeping haemoglobin gene was amplified by PCR and visualized by gel electrophoresis for both control and pathological samples. The DNA quantity was evaluated by a Nano Drop Spectrophotometer (CELBIO). Sodium bisulfite modification of 100µg DNA for each sample was also performed. All methylation-specific PCRs were optimized to detect >5% methylated substrate in each sample. Each experiment was performed in triplicate. In our study RAR-beta represents a frequently un-methylated gene in cancer. In this figure we reported a representative OSCC with methylated promoter of RAR-beta.
Finally, it seems appropriate to conclude this section by reporting synthetically our experimental data. In our laboratory we have analysed, in a series of primary OSCCs with matched normal oral mucosa, the methylation status of a panel of genes, including some of the above mentioned ones and in particular: hMLH1, MGMT, and RAR-beta-2, in order to define an epigenetic fingerprint of the oral cancer (unpublished data). This study would integrate our first results, obtained in a previous published work on methylation status of WNT pathway inhibitors in oral cancer (Pannone, G., *et al.*, 2010). Thirty-seven cases of formalin-fixed, paraffin-embedded OSCC with relative controls of normal oral epithelium were analysed by methylation specific PCR (MSP). Also in this work we have observed different frequencies of gene methylation (Table 1). Characteristically, and in addition to the literature reported data, we have noted that also the healthy oral mucosa shows a

methylated background. In our study population the most frequently methylated gene in cancer was represented by hMLH1(53%), although higher levels of methylation were found in control oral mucosa. RAR-beta-2 (Figure 3) and MGMT (Figure 2) promoter hypermethylation was found in both cases only in 13.5% of OSCCs, but more frequently in healthy oral mucosa (respectively, 28.5% and 23% of studied cases). Therefore, for all genes, logistic multiple regression was performed, in order to verify the association between methylation status of gene promoter (covariates) and presence of cancer (response variable). The Wald test confirmed the statistical significance for RAR-beta-2 ($p=0.044$; CI at 95%).

Similarly to other reviewed and here commented works, this study highlights the importance of epigenetic silencing, showing that a panel of genes may be useful in clinical practice separating normal oral epithelia from the cancerous ones if their DNAs were analysed by methylation specific PCR technique.

All these results not only shed light on a molecular mechanism contributing to OSCC tumorigenesis, but also suggest that employing of an epigenetic fingerprint, together with the classical histo-pathological parameters, may improve the current diagnostic tools, but also contribute indirectly to therapeutics as predictor of choice for the correct clinical management of oral neoplastic and pre-neoplastic lesions.

Variables	Methylation frequency in OSCC (n.37)	Methylation frequency in oral mucosa (n.20)
<i>hMLH1</i>	56%	61%
<i>MGMT</i>	13.5%	23%
<i>RAR-b</i>	13.5%	28.5%

Table 1. **Methylation frequencies in our representative series of OSCCs.**
 Table summarizes data referring to our MSP analysis in oral cancer.

3. RNA interference (siRNA and miRNA)

Cancer is a multi-factorial disease that requires inactivation of tumour-suppressor genes and activation of proto-oncogenes. DNA sequences of these genes are transcribed to mRNA, which is finally translated into functionally aberrant proteins. This may depend on genetic and non-genetic changes, eventually induced by external factor risks. In this frame, RNA is not a ‘passive intermediate product’ between DNA and proteins. The expression of genes is also dependent on RNA-based mechanisms, including nonsense-mediated decay, alternative splicing, RNA editing, and in particular siRNA and micro-RNAs (miRNAs).

3.1 siRNA

Therapeutic use of some siRNAs has been tested in oral cancer both in vivo and in vitro. Some studies correlate siRNA trasfection with OSCC pathogenesis and other features like metastasis, vascular invasion, angiogenesis and cell proliferation. Muramatsu T and Saitoh M (Muramatsu, T.; Saitoh, M., 2008) have studied the expression of syndecan-1 in some oral cancer cell lines (HSC2, HSC3, HSC4, Ca9-22, SAS, KB and BSC-OF) and tested whether

transfection of an siRNA against human syndecan-1 affected the malignant potential of these cells. Syndecan-1 is a prognostic factor in various types of tumors, suggesting its correlation with malignancy and metastasis. Quantitative real-time RT-PCR (QRT-PCR) was carried out and showed that syndecan-1 was expressed in Ca9-22 cells and that it was significantly higher (> 10-fold) than in the other oral cancer cell lines. Transfection of syndecan-1 siRNA was carried out on Ca9-22 cells, which increased their growth rate and their invasive ability.

Nadarajah Vigneswaran and Jean Wu have utilized small interference RNAs (siRNA) to silence cystatin M gene expression in a metastatic oral cancer cell line (MDA-686Ln) expressing high levels of cystatin M, a well known inhibitor of lysosomal cysteine proteinases. By quantitative real-time RT-PCR and Western blotting authors showed that siRNAs were effective in suppressing cystatin M expression by > 50% at both mRNA and protein levels. Cystatin M inhibition significantly increased the enzymatic activities of some lysosomal enzymes both in the media and intracellularly. MDA-686Ln cells treated with siRNA also demonstrated markedly increased proliferation rate, *in vitro* motility and invasiveness. (Vigneswaran, N.; Wu, J., 2006).

Reduced expression of p27 is frequently observed in various cancers including oral squamous cell carcinoma and is due to an enhancement of its ubiquitination, by S phase kinase-interacting protein 2 (Skp2), an F box protein. Overexpression of Skp2 was frequently found in oral squamous cell carcinoma and inversely correlated with p27 expression. Yasusei Kudo and Shojiro Kitajima investigated if small interfering RNA (siRNA)-mediated gene silencing of Skp2 can be employed in order to inhibit p27 down-regulation in oral squamous cell carcinoma. They proved that Skp2 siRNA transfection decreased Skp2 protein and induced the accumulation of p27 protein in oral squamous cell carcinoma cells, interestingly with an important inhibition of the neoplastic cell proliferation both *in vitro* and *in vivo*. These findings suggest that siRNA-mediated gene silencing of Skp2 can be a novel modality of cancer gene therapy for suppression of p27 down-regulation (Yasusei Kudo; Shojiro Kitajima, 2005).

Human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) are considered effective molecular targets for current anticancer therapy. Y Li and M Li investigated the therapeutic effects of targeting and reducing Human telomerase (hTR) and human telomerase reverse transcriptase (hTERT) individually or in combination by recombinant adenovirus-delivered small interfering RNA (siRNA) in oral squamous cell carcinoma (OSCC) Tca8113. The levels of hTR mRNA, hTERT mRNA, hTERT protein and telomerase activity in Tca8113 cells human were heavily reduced, demonstrating that the siRNA-expressing recombinant adenoviruses were an effective anticancer tool for treatment of OSCC.. Furthermore, the mechanism of this anticancer effect in OSCC was not only related to the inhibition of cell proliferation and the induction of cell apoptosis, but might also involve the inhibition of tumor angiogenesis of solely targeting hTR was more direct and efficient, compared with the effect of targeting hTR and hTERT in combination, or hTERT exclusively (Li,Y.; Li, M., 2011).

Finally, ongoing experimental studies are proving the attractiveness and the efficacy of siRNAs as a modern and innovative genetic engineering method.

3.2 miRNA

miRNAs based mechanisms of gene expression regulation could constitute one or more epigenetic steps involved in cancer development (Lu, J.; Getz, G.; 2005). The expression pattern of miRNAs are usually altered in many cancers and appear to be tumor and tissue specific. Several variations of miRNA expression have been identified in oral squamous cell carcinoma tissues/cell lines, and even their plasmatic/salivary levels, when compared to the corresponding normal controls (Gomes, C.C.; Gomez, R.S., 2008).

On these bases, therapeutic use of some miRNAs has been tested *in vitro*. In addition, there are some profiling studies that correlate miRNA expression profiles with OSCC pathogenesis and other features like metastasis, chemo-resistance, prognosis, vascular invasion, alcohol, HPV positivity (Jiang, J.; Lee, E.J., 2005).

3.2.1 Alteration in intracellular, plasmatic, salivary levels

miRNA function regulating gene expression by binding mRNAs and causing degradation of the transcription product or blocking its translation. Therefore, if this process affects tumor suppressor genes or proto-oncogenes, miRNAs may have a key role in carcinogenesis and, potentially, in cancer therapy.

Indeed, in the last ten years the biggest research effort has been put to find out if there were a significant change in miRNA quantity in OSCC tissues, so that we could have used miRNAs as hallmark for early diagnosis; for the same reason, significant changes of miRNAs quantity in OSCC patients have been investigated in serum and saliva. Of the approximately 100 miRNAs identified in OSCC cells, researchers have discovered alteration of cytoplasmic levels only in a small amount, and, among these, in a few cases the meaning of this change is known. Most of the investigated miRNAs show an increase in OSCC cells: miR 21, 24, 31, 155, 181, 184, 211, 345, 375 (Chang, K.W.; Liu, C.J., 2008)

Among these, high level of miR 21, by down-regulating TPM1, PTEN and PDC4, seems to be related to high tumor invasion and poor prognosis (Li, J.; Huang, H., 2009; Reis, P.P.; Tomenson, M., 2010).

Mechanism of function of miRNAs 24 and 181 is known too, targeting the first the RNA binding protein “dead end 1” (DND1) and therefore influencing CDKN1B, and the latter influencing K-RAS expression (Liu, X; Wang, A., 2010; Lin, S.C.; Liu, C.J., 2010; Shin, K.H.; Bae, S.D., 2011). Furthermore, some miRNAs (31, 184) showed high plasmatic level in OSCC, decreasing dramatically after tumor excision (Liu, C.J.; Kao, S.Y., 2010). On the other hand, miRNAs let-7b, 26, 100, 107, 124, 125, 133, 138, 139, 200, 375 decrease in cancer. Decreased cytoplasmic levels of miRNA 124 have been related to a loss of intergrin beta-1 (ITGB1) expression (Hunt, S.; Jones, A.V., 2010); let-7b under-expression causes an over-expression of “Dicer”, an RNAase III endonuclease required for RNA maturation, resulting in increased cell proliferation (Jakymiw, A.; Patel, R.S., 2010).

In saliva, approximately 50 RNAs have been found; among these, miR 125 and 200 showed a statistically significant lower level in OSCC patients. (Lo, W.L.; Yu, C.C., 2011).

3.2.2 Precancerous lesions and risk factors

Some miRNAs have been put in relation with risk factors, chemo-resistance, and malignant progression of lesions. In a very few cases, we know biomolecular basis of this phenomenon.

Cerivigne et al. quantified miR expression changes in leukoplakia and same-site OSCC in 43 sequential progressive samples from 12 patients and four non-progressive leukoplakias from four different patients, and identified a miR signature associated with progression (Cerivigne, N.K.; Reis, P.P., 2009),

These findings were also validated using quantitative RT-PCR in an independent cohort of 52 progressive dysplasias and OSCCs, and five non-progressive dysplasias. The result of the study was that global miR expression profiles distinguished progressive leukoplakia/OSCC from non-progressive leukoplakias/normal tissues. miR-21, miR-181b and miR-345 expressions were consistently increased and associated with increases in lesion severity during progression. They concluded that over-expression of miR-21, miR-181b and miR-345 could play a role in malignant transformation.

Lajer et al. characterized the expression of miRNAs in clinically sampled oral and pharyngeal squamous cell carcinoma (OSCC and PSCC) to describe the influence of HPV, analyzing 51 patients with OSCC/PSCC and 40 control patients with microarray method. HPV positive OSCC patients revealed perturbations of 21 miRNAs, most significantly miR-127-3p and miR363. They concluded that the influence of HPV on miRNA could help understanding the distinct clinical behavior of HPV-infected tumors (Lajer, C.B.; Nielsen, F.C., 2011).

Wald A.I et al. stated that miRNAs differentially expressed in the presence of HPV-16 might provide biomarkers for SCCHN (Squamous cell carcinoma of head and Neck) and identify cellular pathways targeted by the virus (Wald, A.I.; Hoskins, E.E., 2010). Some Authors showed that the miRNAs miR-363, miR-33, and miR-497 were up-regulated, whereas miR-155, miR-181a, miR-181b, miR-29a, miR-218, miR-222, miR-221, and miR-142-5p were down-regulated in HPV-positive cells compared to both HPV-negative SCCHN and normal oral keratinocytes (Liu, X.; Yu, J., 2009).

Moreover, HPV-16 E6 oncogene altered miRNA expression in human foreskin keratinocytes (HFKs) and in an HPV-16-positive cell line with E6 knockdown using siRNA.

Finally, Avissar et al. reported that, in oral and pharyngeal squamous cell carcinoma patients, expression of miR-375 has been shown to increase with alcohol consumption. This shows further damage caused by alcohol, in addition to the many ones already known (Avissar, M.; McClean, M.D., 2009).

3.2.3 New expectations of treatment

Due to their capacity to regulate gene expression, miRNAs may contribute to improve treatment by both representing a new chemotherapy drug both helping to understand mechanisms of resistance to already existing chemotherapy drugs or radiotherapy (Wu, B.H.; Xiong, X.P., 2011).

Researches on therapeutic use of miRNAs are in vitro studies of inhibition of OSCC cell growth after regulating one or more miRNAs expression. Wong et al. report that inhibition of miR-184 could reduce cell proliferation rate and/or induce apoptosis in tongue SCC cell lines, by down-regulation of c-Myc (Wong, T.S.; Ho, W.K., 2009; Wong, T.S.; Liu, X.B., 2008). Henson et al. found that transfecting cells with exogenous miR-125b and miR-100, which are down-regulated in OSCC tumors and cell lines, significantly reduced cell proliferation and modified the expression of target and non-target genes, including some genes that are over-

expressed in radio-resistant OSCC cells. They concluded that the down-regulation of miR-125b and miR-100 in OSCC could play a role in the development and/or progression of disease and may contribute to the loss of sensitivity to ionizing radiation (Henson, B.J.; Bhattacharjee, S., 2009).

Finally, miRNAs may play a role in resistance to cisplatin chemotherapy (Yu, Z.W.; Zhong, L.P., 2010). Yu et al. demonstrated that inducing let-7a miRNA proliferation in head and neck cancer cell lines could significantly inhibit the *stemness* signature and the chemoresistant abilities (Yu, C.C.; Chen, Y.W., 2010).

The development of modified miRNA molecules with longer *in vivo* half lives and efficiency, such as the locked nucleic acid (LNA)-modified oligonucleotides, the anti-miRNA oligonucleotides (AMOs) and the 'antagomirs' is the first step towards bringing these fundamental research advances into medical practice (Orom, U.A., Kauppinen, S., 2006; Weiler, J., Hunziker, J., 2005). Upcoming *in vivo* experiments of miRNA transgenics and knockouts will offer valuable information about safety and efficacy.

4. Meaning of epigenetic and future perspectives

With an annual incidence worldwide of over 500 000 cases, oral squamous cell carcinoma is the eighth most common malignancy today. This epithelial cancer is characterized by the poor outcome, with the surgical margin status as a relevant prognostic factor associated with local recurrence and poor survival. Screening and early detection are believed to decrease both morbidity and mortality associated with OSCC because, unlike many other anatomic sites, oral cavity pre-malignant lesions are often visible upon clinical examination. Oral carcinogenesis is a multi-factorial process involving numerous genetic processes that can alter the function of oncogenes, tumor suppressor genes, and other related molecules. The resulting anomalies can increase the production of growth factors and the number of cell surface receptors, and/or increase transcription or intracellular messenger factor levels. These changes can cause a loss of tumor suppressor activity and give rise to malignant cell phenotypes, able to increase cellular proliferation, weakening cell cohesion, causing local infiltration and metastasis.

Epigenetic phenomena are non-genetic event able to cause modification in gene expression. Such modifications may be passed on successive generations of cells. Under these bases, if one or more oncogenes are directly or indirectly affected by epigenetic changes, malignant cell transformation may occur, because these alterations pass on successive generations of cells, even if there is no mutation in correspondent genes (Esteller, M.; Corn, P.G., 2001).

Cellular aging, risk factors and, as recently discovered, condition of chronic inflammation via mediators such as IL-6 may be potential inducers of epigenetic alterations in oral mucosa cells (Gasche, J.A.; Hoffmann, J., 2011).

We have seen that, among the three possible epigenetic phenomena - DNA hypermethylation, histone code changes and RNA interference, hyper-methylation and miRNAs are the most investigated, and for that reason best understood.

About gene promoter hyper-methylation, p16 and E-cadherin are the most investigated genes, generally statistically significantly hyper-methylated in OSCC. Hall et al. found, in one of the most interesting study, an high correlation between malignant transformation and p16 promoter methylation status (Hall, G.L.; Shaw, R.J., 2008).

As for p16, E-cadherin is highly methylated in malignant lesions but it has not been found, when investigated, a correlation between methylation status and protein expression – in some cases hyper-methylation led to a protein hyper-expression. Inhibitors of WNT pathway methylation promoter status were investigated in a few works, only SFRP 2, 4, 5, DKK2 and WIF 1 were found to be statistically significantly hyper-methylated if compared to healthy tissue. SFRP 1 promoter is less methylated in OSCC according to Pannone et al. hMLH1 promoter methylation investigation produced heterogeneous results (from 0 to 76%) (Pannone, G.; Bufo, P., 2010); still, it is interesting to notice that, in hyper-methylated OSCC, protein expression was reduced in about 30% cases only. MGMT and Rar-beta-2 are generally found hyper-methylated in OSCC. Researches on other genes, such as DCC, RASSF1, KIF1A, NID2, HOXA9 and EDNRB showed promising results, proving that there is still much to investigate (Ogi, K.; Toyota, M., 2002).

What emerges from a critical review of literature data is that methylation status of oncogenes promoters does not seem a valid parameter to predict oncoprotein expression. On the other hand, a methylation profile variation in dysplastic cells cannot be denied. Hyper-methylation of oncogenes promoters in oral mucosa cells emerges as a warning light of ongoing/occurred malignant transformation, concurring to outline a “molecular fingerprint” which can be very helpful in malignancies diagnosis. Still, another most interesting application of epigenetics could be the upstaging of surgical margins. Some works show that the histologically negative surgical margins of OSCC exhibits frequent aberrant DNA methylation changes for number of many cancer-related genes (Sinha, P.; Bahadur, S., 2009). Revealing promoter hyper-methylation present in negative margins could be an useful molecular marker for the poor overall survival (Supic, G.; Kozomara, R., 2011). Surgical excision of the entire affected oral mucosa is not feasible, but the inclusion of more rigorous treatment and more intensive surveillance during follow-up in patients with methylation changes detected in surgical margins may provide an enhanced overall survival.

It is apparent that further studies on larger patients groups and additional quantitative/qualitative validation are needed to understand which one(s) is/are the most significant gene(s).

The analyses of miRNA expression profiles have been found to be useful in the classification and diagnosis of some human tumors (Liu, X.; Chen, Z., 2009). Although the causes of miRNA mis-expression in cancer cells is not understood, it is interesting to note that, in cancer cells, some overexpressed oncogenic miRNAs are located in amplified genomic regions, whereas the down-regulated suppressor miRNAs are located in deleted genomic regions. Over-expression of oncogenic miRNA may reduce protein products of tumor-suppressor genes. On the other hand, loss of tumor-suppressor miRNA expression may cause elevated levels of oncogenic protein. One or both of these alterations could represent new targets for cancer diagnosis and treatment in the future. The demonstration that miRNA expression is related to stage of some tumours may also be a useful tool for prognosis analysis, and it should be evaluated in OSCC staging. In recent years, since researchers have focused on epigenetic alterations in OSCC cells, the emergence of miRNA knowledge and its potential action create new perspectives in understanding cell transformation. The discovery of miRNA, 20-22 nucleotide-long members of the non-coding RNA family, adds another layer of gene regulation that is altered as cancer develops. They

may be present as intergenic transcription units or found in the intronic sequences of protein-coding genes. More than 1,000 of these sequences have been identified until now, and functional studies have identified that miRNAs act as conventional tumor suppressors or as oncogenes, and affect both translation and stability of target mRNA. Most of them are negative regulators of gene expression and have fundamental roles in biologic processes with this function being deregulated as cancer develops, but still, there is much more to understand.

Since distinct miRNA expression profiles vary between OSCC and healthy mucosa, analysis of miRNA expression profiles offers an opportunity for early-stage diagnosis of OSCC, showing a high sensitivity and specificity to classify OSCC. Also, some individual miRNAs have been suggested to be putative specific biomarkers for OSCC diagnosis and prognosis, such as aberrantly over-expressed miR21 (Cervigne, N.K.; Reis, P.P., 2009). In addition, miRNAs seem to hold significant potential as diagnostic tools to detect metastatic disease.

Some miRNAs have been linked with risk factors, chemo-resistance, and malignant progression of lesions (Kumar, M.S.; Lu, J., 2007). In a very few cases, we know biomolecular basis of this phenomena. A small number of miRNAs have been revealed to have profound prognostic values in determining the survival of patients with OSCC. By multivariate analyses, miR-21 expression is proposed as an independent predictor of poor survival for patients with OSCC. Different studies support the idea of a strong association of high expression level of miR21 and significantly decreased 5-year survival in patients with OSCC. Over-expression of miR-21, miR-181b and miR-345 could play a role in malignant transformation (Cervigne, N.K.; Reis, P.P., 2009).

HPV-positive oral-opharyngeal SCCs appear as a distinct entity, different from HPV-negative tumors. There is a strong prevalence in younger patients without sex predilection. Up to 20% of these cancers develops in patients without traditional risk factors, i.e. smoking and alcohol abuse. Conversely, their risk factors include young age at first intercourse, promiscuity, and history of genital warts in men and number of sexual partners in women. As positive personal history of oral-genital and oral-anal sexual contacts (during which the HPV infection may be transmitted to the oral cavity) increases the risk for HPV-positive HNSCCs, they may be regarded as a sexually transmitted disease. It is assumed that long-lasting oral HPV infection, which prevalence increases after the onset of sexual activity, precedes the development of HPV-positive HNSCC for about 10 years. In addition, these tumors seem to be related to immune-suppression. HPV positive OSCC patients revealed perturbations of 21 miRNAs, most significantly miR-127-3p and miR363. It has been hypothesized that the influence of HPV on miRNA could help understanding the distinct clinical behavior of HPV-infected tumors (Liu, X.; Yu, J., 2009). In oral and pharyngeal squamous cell carcinoma patients, over-expression of miR375 has been put in relation with alcohol consumption. This shows further damage caused by alcohol, in addition to the many ones we already know (Avisar, M.; McClean, M.D., 2009).

Moreover, low expression levels of miR205 was found to significantly correlate with loco-regional relapse of OSCC, independent of disease severity at diagnosis and treatment, and miRNA expression level seems to change with different malignancy grades and reflect the risk of OPL16 or the biological characteristics of OSCC such as the metastatic potential and chemo-sensitivity or chemo-resistance (Shiiba M.; Uzawa K., 2010).

An increasing amount of studies show that miRNAs circulate stably in body fluids, with different expression pattern in blood and saliva, of healthy and cancer patients. Most of investigated miRNAs show an increase in OSCC cells: miR 21, 24, 31, 155, 181, 184, 211, 345, 375. On the other hand, miRNAs let-7b, 26, 100, 107, 124, 125, 133, 138, 139, 200, 375 seem decreased in OSCC cells (Henson, B.J.; Bhattacharjee, S., 2009; Liu, X.; Jiang, L, 2009).

Elevated levels of miRNA in plasma (31, 184) have been detected in OSCC patients compared with case controlled individuals. Indeed, some miRNAs showed high plasmatic level in OSCC, decreasing dramatically after tumor excision. Saliva, easy to collect, is an ideal medium for clinical applications. About 50 miRNAs were revealed to be present in both whole saliva and supernatant saliva of patients with OSCC; among these, miR 125 and 200 showed a statistically significant lower level in OSCC patients versus healthy controls. Another study showed that over-expressed miR31 was also detectable in the saliva of OSCC patients. These circulating miRNAs seem to be released from the OSCC tissues into the bloodstream, causing the remarkable reduction of plasmatic miRNAs in patients after surgical excision of the tumor. All these data support the view that circulating miRNAs could be used as non-invasive and powerful OSCC biomarkers (Liu, C.J.; Kao, S.Y., 2010).

Due to their ability to regulate gene expression, miRNAs may contribute to improve treatment by both representing a new chemotherapy drug both helping to understand mechanisms of resistance to already existing chemotherapy drugs or radiotherapy.

We need further evidence to understand miRNAs role as either oncogenes or tumor suppressors regulating key genes, involved in the initiation and progression of human cancer. If this role would be definitively proved, this would provide the rational basis for miRNA-based cancer therapy. Up-regulating the expression of tumor suppressive miRNAs at low levels in OSCC as well as inhibiting the expression of oncogenic miRNAs over-expressed is the effective approach for the therapeutic purpose. Inhibition of miR184 could reduce cell proliferation rate and/or induce apoptosis in tongue SCC cell lines, by down-regulation of c-Myc (Liu, C.J.; Kao, S.Y., 2010). It has been found that transfecting cells with exogenous miR125b and miR100, which are down-regulated in OSCC tumor and cell lines, significantly reduce cell proliferation and modified the expression of target and non-target genes, including some that are over-expressed in radio-resistant OSCC cells. Some Authors concluded that the down-regulation of miR 125b and miR100 in OSCC could play a role in the development and/or progression of disease and may contribute to the loss of sensitivity to ionizing radiation (Henson, B.J.; Bhattacharjee, S., 2009; Lo, W.L.; Yu, C.C., 2011; Wong, T.S.; Ho, W.K., 2009; Wong, T.S.; Liu, X.B., 2008).

Finally, miRNA may play another role in resistance to cisplatin chemotherapy. It has been demonstrated that inducing let-7a miRNA proliferation in head and neck cancer cell lines could considerably inhibit the *stemness* signature and the chemo-resistant abilities (Yu, Z.W.; Zhong, L.P., 2010; Yu, C.C.; Chen, Y.W., 2010).

However, at present, it is still a big challenge to design a specific and efficient drug delivery system for miRNA-based drugs.

The discovery of miRNAs provides new insights into the pathogenesis and progression of OSCC, which was thought to be a disease characterized exclusively by alterations in

oncogenic and tumor suppressive protein-coding genes. A number of aberrantly expressed miRNAs have been verified either as oncogenes or tumor-suppressors, participating in various biological processes of OSCC, including proliferation, apoptosis, metastasis and chemoresistance. In addition, these mis-expressed miRNAs have been proved to have potential as diagnostic and prognostic tools. Furthermore, the role of miRNAs in cancers makes it possible to design miRNA-based therapy for OSCC. Although still little is known in this field, compelling evidence gives exciting promises that miRNAs will improve the management of OSCC in the near future. Further studies are needed to generate additional information about tumour-suppressor miRNAs and oncogenic miRNAs involved in OSCC pathogenesis, including oral pre-malignancies transformation (Clague, J.; Lippman, S.M., 2010).

5. Summary

The purpose of this chapter was to review the current state of knowledge of the genetic/epigenetic alterations that are specifically observed in oral mucosal pre-malignancy and cancer. The ultimate goal of research on OSCC is to identify the specific candidate biomarkers that would have optimal predictive capacity in identification of those dysplastic lesions most likely to progress to OSCC over time, pointer towards the right therapy, help to better define surgical margins.

Taking a critical look, we must highlight how carcinogenesis is a complex phenomenon, involving a wide pool of genes which expression can be modified by an astonishing amount of factors. On the other hand, an epigenetic methylation/histonic/miRNA profile variation in dysplastic cells cannot be denied. These changes in oral mucosa cells emerge as a warning light of ongoing/occurred malignant transformation, concurring to outline a “molecular fingerprint” which can be very helpful in malignancies diagnosis. It appears that the best way to understand hyper-methylation phenomenon role in carcinogenesis should not be exclusively investigating its frequency in OSCC cells – always comparing results to healthy mucosa from OSCC patients and healthy mucosa from healthy patients, but also monitoring pre-malignant oral lesions, establishing a correlation between this epigenetic event and malignant transformation.

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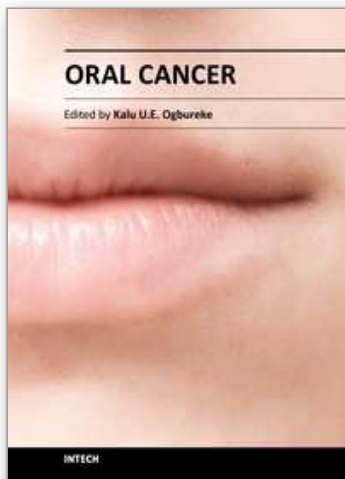
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Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

How to reference

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