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MicroRNA in Oral Squamous Cell Carcinoma and Oral Potentially Malignant Lesions: from biological discovery to clinical utility.

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Introduction

1.1 Oral Squamous Cell Carcinoma (OSCC)

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity. It represents the majority of head and neck cancers with more than half million patients being affected each year worldwide [1]. More than 90% are squamous cell carcinomas which are mostly attributed to exogenous factors such as tobacco smoking and heavy alcohol consumption. Advances in cancer research have provided new information on the cellular and molecular processes in carcinogenesis. This also has lead to the identification of biological markers and effective treatment options. The long-term survival rates, however, remain low and many individuals are affected.

1.1.1 OSCC epidemiology

OSCC is the eighth most common cancer in the world, with the highest prevalence among men (5-year prevalence in men: 401,075) [2]. According to Ferlay et al. the worldwide cases of oral cancer in 2008 in both sexes were about 263,000 (2.1% of the total cancers), the incidence rate was 3.9 per 100,000 persons and approximately 127,000 cases were fatal. According to the American Cancer society the incidence of OSCC is higher in developed countries when compared to developing countries, but the mortality rates remain higher in developing countries. In developing countries the incidence of OSCC is 107,700 in males and the estimated deaths are 61,200 [3].

In south-central Asia, OSCC is one of the third most frequent types of cancer. In India the incidence rate is 12.6 per 100 000 population, and in other countries of Asia OSCC remains one of the most common cancers [4, 5]. Of interest, the incidence rate remains high in

several developed countries such as Denmark, Poland, Germany, Scotland, and also in Australia, Japan, New Zealand and the USA [6, 7].

1.1.2 Survival Rates

The 5-year survival rate has been relatively low for OSCC despite advancement in diagnosis and treatment. According to the Surveillance, Epidemiology and End Results Program the overall 5-year relative survival is 62.2%. The 5-year survival rate of late-stage OSCC (distant, cancer has metastasized) is only 20% and it is approximately 82% for early stage OSCC (localized tumor, confined to primary site) [8]. In USA from 1983 to 2006, the five-year survival rate has increased from 52.5% to 60.8% within the time period [9]. Data from the World Health Organization showed a similar negative trend in the survival rates between 2005-2010 in some countries (e.g., Brazil, Egypt, Germany, Japan, Netherlands, Poland, United Kingdom) [10] where the number of deaths has increased.

1.1.3 Demographic and Anatomical sites

OSCC arises from mucosa lining of the oral cavity or from the lips. The most common type is squamous cell carcinoma, and the histological grade can vary from well-differentiated keratinizing to undifferentiated non-keratinizing with a high tendency to metastasize. In the United States the median age at diagnosis for cancer of the oral cavity is 64.5 years of age [11]. The tongue remains the main site of OSCC [12-16], affecting particularly the lateral posterior border, in older males individuals [17]. Interestingly, a new trend emerged during the last 20 years; the rate of OSCC (especially tongue cancer) increased in younger patients without any apparent and common risk factor such as tobacco or alcohol consumption [18, 19]. The increased trend of OSCC in younger patients merits further investigation. Data from 2006-2010 show that the total percentage of cancer of the tongue

who occurred in people younger than 45 years old is 7.5% whereas the median age at diagnosis for tongue cancer is 61 years of age [20]. The other most involved sites are the lips (17%) and the floor of the mouth (14%). Lip cancer, especially the lower lip, is typically observed in people who are exposed to sunlight (e.g., fishermen, farmers, skiers and windsurfers) [21].

1.1.4 Diagnosis

The clinical appearance of OSCC is variable and requires an expert eye to recognize its features. Early lesions may appear as red oral mucosa failing to heal within two weeks, or as a persistent lump with spontaneous bleeding or ulceration [22]. Lesions may appear flat, raised, exophytic or ulcerated without any initial symptoms. Over time patients may complain of difficulties chewing, limited tongue movement or an abnormal sensation secondary to swelling. After the cancer growth, more symptoms occur and include bleeding, paresthesia, mobile teeth (when the tumor invades the bone), and induration and fixation of soft tissues; only one third are diagnosed with localized tumors [23]. Any suspicious and persistent lesion should be followed up by the clinician and biopsied [24].

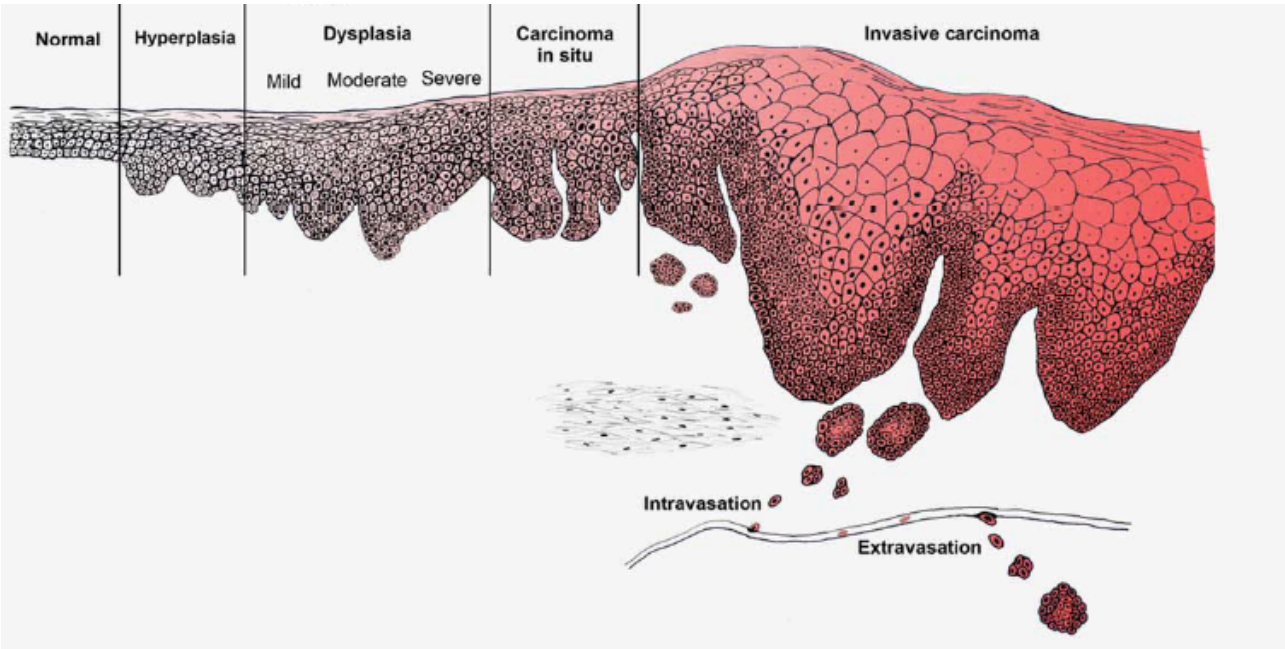
1.1.5 The malignant progression

Normal cells transform into preneoplastic cells and then to cancer after a series of clinical and histopathological stages involving genetic and molecular changes. These stages are clinically represented by manifestations on oral mucosa, such as leukoplakia, erythroplakia or leukoerythroplakia, and they all represent a predictive factor of malignant transformation [25].

The multi-step progression of cancer involves a combination of acquired and inherited alterations in the DNA sequence. Genetic changes in keratinocytes cause a progressive

acquisition of a malignant phenotype from premalignant to cancer, characterized by invasion across the epithelial basement membrane and eventual metastasis. The overexpression of oncogenes causes a disruption in the cell cycle driving to abnormal cell proliferation [2], while the expression of the tumor suppressor genes, especially the proteins p53 and p16 in the dysplastic epithelium are significant markers to detect potentially malignant lesions in the oral cavity [26].

Risk factors can lead to genetic and epigenetic alterations; the most observed cases of mutation of these genes are present in people from Asia due to the tobacco chewing and betel quid [27, 28]. Furthermore, epigenetic may cause an alteration of gene expression through aberrant DNA methylation, histone modifications and expression of microRNAs [29].



Dionne KR, Warnakulasuriya S, Zain RB, Cheong SC. Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. Int J Cancer. 2015 Feb 1;136(3):503-15.

1.1.6 Oral potentially malignant lesions (OPML)

OPML comprise leukoplakia, erythroplakia, oral lichen planus and oral submucous fibrosis. These lesions are characterized by sequential accumulation of molecular changes that can lead to dysplasia (mild, moderate or severe) and then to frank invasive carcinoma [30].

Oral Lichen Planus (OLP) is an immuno-mediated inflammatory condition of the oral mucosa [31]. It occurs in 1 to 2 % of adults and may be idiopathic or associated with a variety of systemic and local conditions. OLP usually affects the buccal mucosa and tongue bilaterally, and can present with three distinct forms: reticular/keratotic (classic), erosive/erythematous, and ulcerative forms. Less than 1% of OLP evolve in OSCC [32, 33].

Oral Leukoplakia (OL) is a white lesion that can affect any site of the oral cavity, and its diagnosis it is made by the exclusion of the other known diseases. The malignant transformation rate of all leukoplakias is 9-37%. There are three clinical different type of leukoplakia (the homogeneous, the non homogeneous and the verrucous type); the most aggressive is the proliferative verrucous type (60-100% of proliferative leukoplakias develop carcinoma) [34]. The risk of malignant transformation is meanly correlated to the degree of histological dysplasia (mild, moderate or severe) that represents the histological step of the epithelial malignant transformation. However, dysplasia has limited prognostic value. Nowadays, there are not specific markers that can predict the probability of malignant progression from dysplatic lesions to cancer. Some OL can transform into cancer after a series of progressing genetic alterations. OL that transformed into OSCC are called progressive OL. The multi-step progression involves a combination of acquired and inherited alterations in the DNA sequence that can lead to OSCC.

According to the WHO definition oral erythroplakia is defined as “any lesion of the oral mucosa that presents as bright red velvety plaques which cannot be characterized clinically or pathologically as any other recognizable condition”. The risk of malignant

transformation of erythroplakia is the highest between the others premalignant forms (90%). This lesion presents as red plaques that can be depressed or flat, and they occur mainly on the floor of the mouth, the soft palate and the ventral tongue [35].

Oral submucous fibrosis is a condition characterized by a fibrous aspect, a significant morbidity with pain and reduced oral opening which may affect any site of the oral cavity [36]. It is associated with areca nut chewing especially in Southeast Asia and the reported risk of malignant transformation varies from 2.3-7.6% [37].

1.2 MicroRNAs

Mature microRNAs (miRNAs) are short, single-stranded noncoding RNAs of 21–24 nucleotides in length that regulate gene expression post-transcriptionally by degrading or repressing mRNA. Specifically, miRNAs associate to their target mRNAs by base-pairing to partially complementary sites, usually located in the 3'untranslated region (3'UTR) [38, 39]. A single miRNA can regulate the translation of multiple genes, thus it can modulate the expression of multiple proteins.

MiRNAs can influence numerous signaling cascades and biological networks, including cell proliferation, differentiation, development, metabolism, apoptosis, migration, senescence and response to stress.

MiRNAs play an important role in cancer because they regulate the expression of tumor-suppressors genes and oncogenes. In fact, miRNA expression profiles are often altered in malignant cells, being down- or up-regulated when compared with those in normal tissues. The identification of miRNAs that have an oncogenic-like effect and their signature for every specific cancer represent a novel challenge in cancer research.

A comprehensive understanding of the role of miRNAs during the pathogenesis of cancer, and the identification of miRNA profiles specific for all tumors, will offer new opportunity for diagnostic, prognostic, and therapeutics.

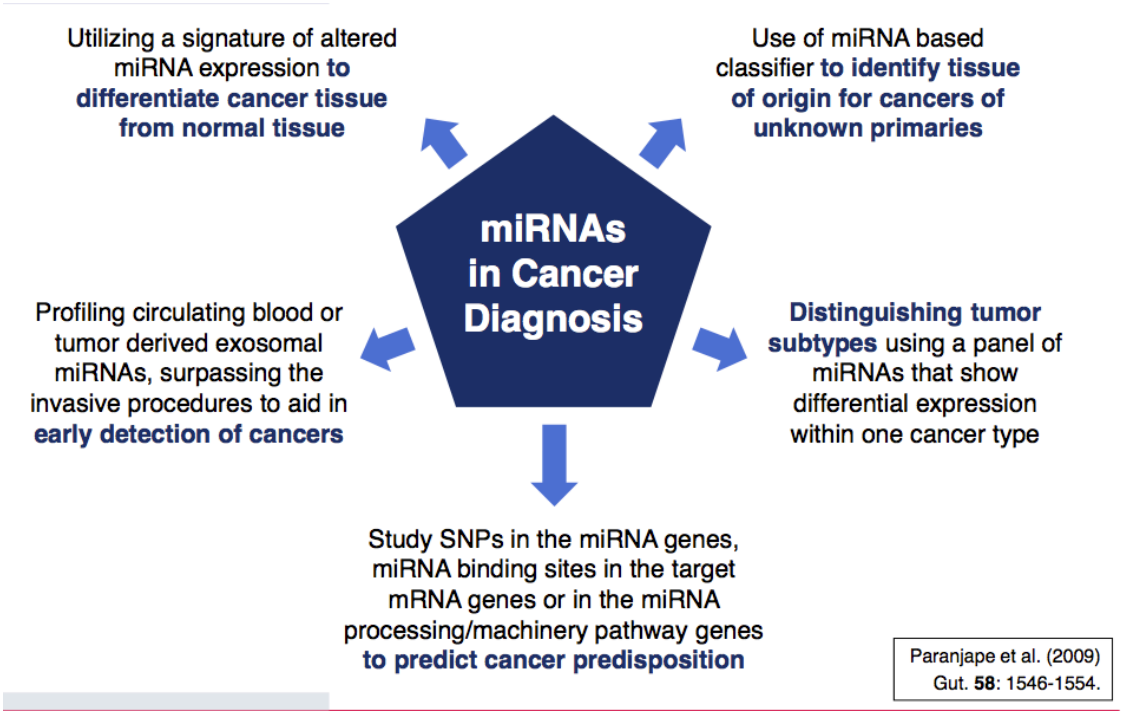
1.2.1. MiRNA biogenesis and role

The biogenesis of miRNAs begins with the transcription inside the nucleus of the pri-miRNA by the RNA polymerase II [40]. The pri-miRNA then is cropped by the microprocessor complex into a 70 nt hairpin called pre-miRNA [41]. The pre-miRNA is then exported in the cytoplasm where it is cleaved by the RNAse Dicer in two short sequences of nucleotides and one of them is incorporated into the RNA-induced silencing complex (RISC) [42, 43]. The mature miRNA targets the mRNAs via sequence complementarity, resulting in the degradation or translation repression of the mRNA. MiRNAs act as negative regulators of gene expression and are involved in tumorigenesis by targeting tumor-suppressor genes or oncogenes directly or indirectly. In general, miRNAs are differently and abnormally expressed in cancer cells compared to normal tissues, and this different expression is correlated to the development of the tumor, in particular it is correlated to the expression or deletion of genes involved in tumor growth [44].

The identification of miRNAs that are potential tumor-suppressors (tumor-suppressor miRNAs) or oncogenes (oncogenic miRNAs) may lead to the discovery of a new class of potential biomarkers for tumors. For example, a loss of expression of tumor-suppressor miRNAs may lead to elevated levels of the protein products of target oncogenes, and the overexpression of oncogenic miRNAs may reduce the levels of protein products of target tumor-suppressor genes [45]. Oncogenic miRNAs are overexpressed in the initial phases

of the tumor growth and their number increases during the progression of the tumor, while tumor-suppressor miRNAs are under-expressed.

The expression profiles of miRNAs that act as tumor-suppressors are usually altered in many cancers. Their reduction accelerates the oncogenic transformation through the deregulation of target oncogenes, while their overexpression indicates that miRNAs can halt the cancer cell growth, or induce apoptosis [46].



1.2.2. MiRNA in OPML

Xiao et al. reported 24 over-expressed and 9 under-expressed miRNAs in 7 malignantly transformed OLs compared to 20 non-progressive OLs using human oral leukoplakia cell line [47]. Yang et al. found 12 over-expressed and 13 under-expressed miRNAs in 8 progressive OLs compared to 7 non-progressive OLs in saliva samples [48]. De Sarkar et al. showed that only miR-31 is over-expressed both in gingival buccal SCC and OLs using

samples from biopsies [49]. However, the most consistent article that describes a miRNA signature associated to progressive OPML is the study of Cervigne et al. [50]. They examined the miRNA expression in 29 OLs that progressed to OSCC compared to 4 non-progressive OLs and 7 normal tissues, and they found a large number of altered miRNAs. In particular, miR-21, miR-181b and miR-345 showed an increased expression during the progression to OSCC. The authors showed that some of the genetic alterations in OSCC are earlier expressed in the same-site premalignant lesion. Moreover, the role of miR-345 is still not well understood, however it seems to under-expressed the BAG3 expression, an anti-apoptotic molecule. MiR-345 was over-expressed both in progressive OLs and OSCC.

1.2.3. MiRNA in OSCC *and metastasis*

MiRNAs can be used as prognostic factors in patients with OSCC. In fact, the TNM staging is not always accurate, thus it is important to correlate biomarkers, such as miRNAs, to the prognosis of patients with cancer. MiRNAs could also be used as biomarkers for the invasive behavior of OSCC. Some articles described the expression of tumor-suppressor miRNAs: miR-138 is under-expressed in cell culture of OSCC metastasis; and miR-491-5p was found under-expressed in invasive OSCC cells, suppressing migration, invasion and metastasis of OSCC cells. Thus, MiR-491-5p is found decreased in advanced OSCC and acts as a metastatic suppressor [51].

The role of oncogenic miRNAs is well described in novel findings: oncogenic miR-181 is increased in lymph node metastasis and vascular invasion enhancing cell mobility; miR-211 is over-expressed in nodal metastasis and vascular invasion of OSCC with poor prognosis [52, 53].

Furthermore, miR-134 was associated with nodal metastasis and mortality of patients with oral cancer; high expression of miR-196a was associated with tumor recurrence, nodal

metastasis, and mortality; a significant association was found between miR-126 expression and tumor progression, nodal metastasis, vessel density, and poor prognosis in OSCC cases [54]. MiR-21 is an established oncogenic miRNA that targets tumor-suppressing genes TPM1 and PTEN [55]. Mir-21 was found over-expressed in SCC of the tongue, and in progressive oral OLs. This oncogenic miRNA promotes the tumor invasion of SCC of the tongue via the Wnt/β-catenin pathway by targeting tumor suppressor DKK2 [56]. MiR-21 was also involved in EMT in human bronchial epithelial cells and hepatocytes, while miR-375 facilitates EMT in cervical cancer cells and breast cancer cells [57, 58].

1.3 N-glycosilation gene *DPAGT1*

Protein N-glycosylation is one of the most abundant posttranslational modifications in biology. N-glycosylation controls a broad spectrum of cellular functions that are vital for development and homeostasis, including cell– cell and cell– matrix adhesion, cell proliferation, cell survival and immune system responses [59]. Dysregulated N-glycosylation is a common theme in disease, including a prevalent association with cancer. In tumors of epithelial origin (carcinomas), cancer progression is associated with dramatic changes in cell– cell or E-cadherin-mediated adhesion, as well as aberrant organization of cell polarity and cytoskeleton architecture [60].

N-Glycosylation is initiated in the endoplasmic reticulum (ER) by the dolichol phosphate-dependent N –acetylglucosamine 1-phospho-transferase (GPT), encoded by the *DPAGT1* gene [61]. Cell– cell adhesion directs cytoskeletal dynamics to establish and maintain cellular polarity, which ultimately determines accurate tissue architecture. Dysregulated cell adhesion is a hallmark of many diseases, most notably cancer. Interestingly, modification of key adhesion and cytoskeletal-regulating proteins with N -glycans controls their activity, and thus dysregulated N-glycosylation may contribute to altered

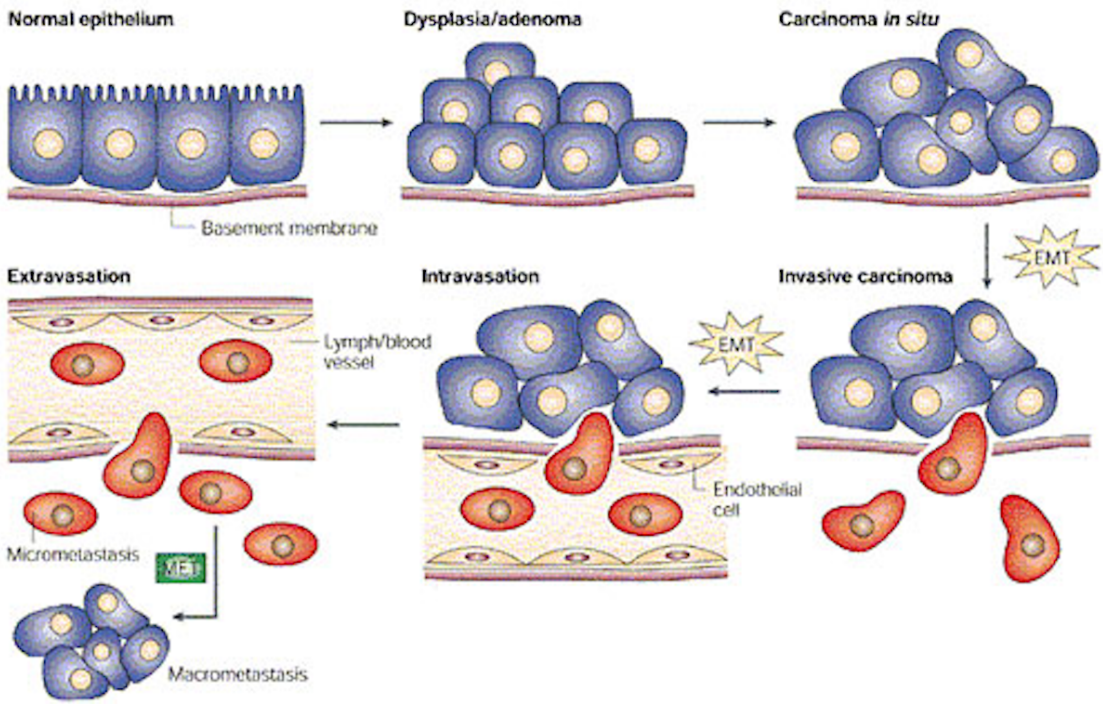
adhesive properties observed in disease [62].

Many epithelial cancers display loss of E-cadherin, and E-cadherin is commonly downregulated in tumors by transcriptional repressors such as SLUG, SNAIL, TWIST and ZEB [63]. However, in some cancers, notably a major subset of OSCCs, E-cadherin is not lost. Rather, due to overexpression of *DPAGT1*, E-cadherin is highly modified with complex N -glycans and unable to form mature cell– cell contacts [64]. This suggests that in OSCC, and most likely in other epithelial tumors that maintain E-cadherin expression, N-glycosylation is a key determinant of reduced E-cadherin adhesion.

1.4 Epithelial-Mesenchymal Transition (EMT)

The epithelial-mesenchymal transition (EMT) refers to a series of biological events that allows epithelial cells to lose their differentiated features, including cell-cell adhesion, planar, apical-basal polarity and lack of motility, and assume mesenchymal phenotypes, including enhanced motility, invasiveness, elevated resistance to apoptosis, and increased production of extracellular matrix (ECM) components [65]. EMTs can be induced by a variety of signaling pathways that converge on a few key families of transcription factors, including the zinc-finger protein Snail (Snail/Slug), ZEB (ZEB1/ZEB2), and basic helix-loop-helix factors, such as Twist [66]. Activation of these transcription factors leads to the repression of E-cadherin as well as other cytoskeletal and cell-surface proteins. The final stages of the EMT are characterized by downregulation of E-cadherin and cytokeratin and up-regulation of mesenchymal-specific markers, such as vimentin and fibronectin [67]. Specific miRNAs have been described with the regulation of EMT. In particular the miR-200 family targets transcription factors ZEB1 and ZEB2. As ZEB factors are EMT inducers, the consequence of miR-200 overexpression is the reduced expression of ZEB factors and subsequent epithelial differentiation [68]. Also miR-205 was found downregulated in cells

that had undergone EMT [69]. Together these miRNAs regulate the expression of ZEB factors. The inhibition of the miRNA could be sufficient to induce EMT. In OSCC the miR-200c expression was found significantly decreased in the regional lymph node metastasis and the miRNA have the ability to block the tumourigenicity and invasion of OSCC cell lines [70]. Together miR-200c and miR-205 are associated with poor prognosis of OSCC [71]. On the other hand, the ectopic expression of these miRNAs could initiate the Mesenchymal to Epithelial Transition [72] in node metastasis reprogramming the gene expression and the formation of adherent junctions.



2. Aim of the research

The aim of the research was to identify the expression profiles of miRNAs in progressive and non-progressive OPML as well as compare miRNA different expression in metastatic

OSCC versus non-metastatic OSCC. Furthermore, the study investigates which miRNAs is related to the development of lymph node metastasis and their expression in oral presurgical biopsies. Moreover, the research wants to investigate the role of miRNAs in OSCC cell lines, especially related to N-glycosilation gene *DPAGT1* and EMT.

3. Materials and Methods

3.1 MiRNA in OPML and OSCC

3.1.1 Case selection

The samples were obtained from the files of Anatomic Pathology Section at the Bellaria Hospital of the Department of Biomedical and Neuromotor Sciences of the University of Bologna were retrieved. All tissue samples were formalin-fixed paraffin-embedded (FFPE). The following cases were selected for the present study:

- 26 non-metastatic OSCC of which 19 of females patients and 7 of males with a mean age of 69 years. Five tumors were in the buccal mucosa, 3 in the floor of the mouth, 9 in the gingiva, 9 in the tongue. Nine OSCC were well differentiated, 10 were moderately differentiated, 7 were poorly differentiated. T1 tumors were 14, and T2 were 12. Presurgical biopsies of the tumors were obtained prior to the surgical intervention.

Case	Age / Sex	Site	Diagnosis	Follow-up
1	93/F	Buccal Mucosa	WD-OSCC	T2N0
2	66/F	Gingiva	WD-OSCC	T1N0
3	50/M	Buccal Mucosa	MD-OSCC	T1N0

4	59/F	Tongue	WD-OSCC	T1N0
5	70/F	Buccal Mucosa	PD-OSCC	T1N0
6	74/F	Gingiva	PD-OSCC	T1N0
7	59/M	Floor of the mouth	PD-OSCC	T1N0
8	64/F	Tongue	PD-OSCC	T1N0
9	64/F	Buccal Mucosa	WD-OSCC	T2N0
10	91/M	Gingiva	WD-OSCC	T2N0
11	67/M	Gingiva	WD-OSCC	T2N0
12	76/M	Tongue	WD-OSCC	T2N0
13	74/F	Gingiva	PD-OSCC	T1N0
14	89/F	Tongue	WD-OSCC	T2N0
15	81/F	Gingiva	MD-OSCC	T2N0
16	52/F	Gingiva	MD-OSCC	T2N0
17	87/F	Gingiva	MD-OSCC	T2N0
18	65/F	Floor of the mouth	MD-OSCC	T1N0
19	63/F	Tongue	MD-OSCC	T1N0
20	35/F	Tongue	MD-OSCC	T1N0
21	51/M	Tongue	MD-OSCC	T1N0
22	72/F	Tongue	WD-OSCC	T1N0
23	79/F	Buccal mucosa	MD-OSCC	T1N0
24	76/F	Floor of the mouth	PD-OSCC	T2N0
25	65/F	Tongue	WD-OSCC	T2N0
26	69/M	Gingiva	MD-OSCC	T2N0

- 26 metastatic OSCC of which 13 females patients and 13 males with a mean age of 68 years. Four tumors were in the buccal mucosa, 1 were in the palate, 6 were in the gingiva, 15 were in the tongue. Three OSCC were well differentiated, 16 were moderately differentiated, 7 were poorly differentiated. T1 tumors were 6, and T2 were 20. All of them were metastatic (N1 24, N2 2 cases). Presurgical biopsies of the tumors were obtained prior to the surgical intervention.

Case	Age / Sex	Site	Diagnosis	Follow-up
1	71/F	Tongue	WD-OSCC	T2N1
2	60/M	Buccal Mucosa	PD-OSCC	T2N1
3	69/F	Gingiva	MD-OSCC	T2N1
4	70/M	Tongue	WD-OSCC	T2N1
5	79/F	Buccal Mucosa	MD-OSCC	T2N1
6	79/M	Tongue	PD-OSCC	T2N1
7	68/M	Tongue	PD-OSCC	T1N1
8	76/F	Palate	MD-OSCC	T2N1
9	70/F	Gingiva	MD-OSCC	T2N1
10	81/F	Gingiva	PD-OSCC	T2N1
11	38/M	Tongue	MD-OSCC	T1N1
12	85/F	Tongue	MD-OSCC	T2N1
13	89/F	Tongue	MD-OSCC	T2N1
14	72/F	Gingiva	MD-OSCC	T1N1
15	70/M	Gingiva	MD-OSCC	T2N1
16	48/M	Tongue	MD-OSCC	T2N1
17	49/F	Tongue	PD-OSCC	T2N1
18	71/M	Tongue	PD-OSCC	T1N1
19	83/F	Gingiva	MD-OSCC	T1N1
20	60/M	Tongue	MD-OSCC	T1N2
21	71/M	Tongue	MD-OSCC	T2N1
22	71/M	Tongue	PD-OSCC	T2N1
23	75/F	Buccal Mucosa	WD-OSCC	T2N1
24	39/M	Buccal Mucosa	MD-OSCC	T2N1
25	74/F	Tongue	MD-OSCC	T2N1
26	48/M	Tongue	MD-OSCC	T2N2

Results were compared with 12 normal oral mucosa specimens collected during tooth extraction from non-smoker healthy patients. All information regarding the human material used in this study was managed using anonymous numerical codes.

- 12 non-progressive OPML from 12 patients (6 female and 6 male patients). Four were in the tongue, 4 from the buccal mucosa, 3 from gingiva, and 1 from the floor of the mouth. Seven OPML were low-grade dysplasia, 5 were moderate dysplasia.

Case	**Age / Sex**	**Site**	**Diagnosis (dysplasia)**	**Follow-up**
1	69/M	Buccal Mucosa	Low grade	T0N0
2	85/F	Tongue	Moderate grade	T0N0
3	90/M	Floor of the mouth	Moderate grade	T0N0
4	74/F	Buccal Mucosa	Low grade	T0N0
5	63/F	Gingiva	Low grade	T0N0
6	52/M	Tongue	Moderate grade	T0N0
7	79/F	Buccal Mucosa	Moderate grade	T0N0
8	67/F	Gingiva	Low grade	T0N0
9	73/M	Gingiva	Low grade	T0N0
10	90/F	Tongue	Low grade	T0N0
11	42/M	Buccal Mucosa	Low grade	T0N0
12	53/M	Tongue	Moderate grade	T0N0

- 7 progressive OPMLs (4 female and 3 male patients). One was in the tongue, four from the buccal mucosa, two from gingiva. One OPML was low-grade dysplasia, 3 were moderate dysplasia, 3 were high grade dysplasia.

Case	**Age / Sex**	**Site**	**Diagnosis (dysplasia)**	**Follow-up**
1	68/F	Buccal Mucosa	Moderate grade	T2N1M0
2	65/F	Gingiva	Low grade	T1N2M0
3	80/F	Tongue	Moderate grade	T3N1M0

4	67/M	Buccal Mucosa	Moderate grade	T1N0M0
5	87/M	Gingiva	High grade	T1N0M0
6	61/F	Buccal Mucosa	High grade	T1N0M0
7	70/M	Buccal Mucosa	High grade	T2N0M0

Results were compared with 5 normal oral mucosa specimens collected during tooth extraction from non-smoker healthy patients.

3.1.2 Statement of ethics

All patients signed a written consent for molecular analysis and anonymous data publication for scientific studies, and all information regarding the human material used in this study was managed using anonymous numerical codes.

3.1.3 MiRNA extraction

Haematoxylin and eosin (H&E) sections from FFPE specimens were reviewed by a pathologist to select the more informative block. Four 20 μm-thick sections were cut followed by one H&E control slide. The tumor area selected for the analysis was marked on the control slide to ensure, whenever possible, greater than 90% content of neoplastic cells (avoiding necrosis and lymphocytes, normal epithelia). The four 20 μm-thick sections were manually dissected under microscopic guidance according to area selected on H&E and incubated in xylene for 3 minutes at 50°C and, after two rinses with ethanol, miRNAs were extracted using High Pure miRNA Isolation kit (Roche, Mannheim Germany), according to manufacturer's instructions. Quality and quantity of smallRNAs extracted from FFPE-dissected tissue were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Qubit fluorometer (Invitrogen, Carlsbad,

CA, U.S.A.). cDNA was obtained after a polyadenylation step and retrotranscription were performed using SuperScript III RT enzyme and a Universal RT Primer according to NCode miRNA first-strand cDNA synthesis and qRT-PCR Kit protocol (Invitrogen, Carlsbad, CA, U.S.A.). Fourteen miRNAs were selected for analysis, according to their role in cancer and data previously published in literature. MiR191 and RNU44 were used as endogenous controls.

Each forward primer used corresponds to mature miRNA sequence according to miRBase database (http://microrna.sanger.ac.uk). Primers were modified with LNA (Locked Nucleic Acid) substitutions for increasing specificity and discriminating between miRNAs with a single base different nucleotide sequences. Universal reverse primer was provided by NCode miRNA first-strand cDNA synthesis and qRT-PCR Kit (Invitrogen, Carlsbad, CA, U.S.A.).

Efficiency of each primer was tested by Real-Time PCR using serial dilutions (1:1, 1:25, 1:50, 1:100) of a pool of RNA extracted by following cell lines: U-87 MG, MCF7 and LNCaP. A run of Real-Time PCR using as template a pool of female DNA (Promega, Madison, WI, U.S.A.) was performed to confirm that miRNAs primers were not able to amplify DNA. MiRNAs expression was evaluated using a AB7000 machine (Applied Biosystem, Foster City, CA, USA) and FastStart Taq Reagents Kit (Roche, Mannheim, Germany), with the following program: 2 minutes at 50°C, 4 minutes at 95°C and 37 cycles with annealing at 60°C for 30 seconds. GelStar stain (Lonza Bioscience, Rockland, ME, USA) was used as Real-Time detector. No template control for each miRNA was included in the reaction plate. All the reactions were performed in duplicate.

The list of analyzed miRNA in FFPE specimens is the following: Let 7d, 7, 10a, 10b, 17, 20a, 21, 26a, 31, 34a, 101, 137, 146a, 181b, 182, 196a, 200a, 200c 3p, 205 5p, 206, 221, 222, 345, 375, 518b, 519d, 520g, 649.

3.1.4 Statistical analysis

Expression values and fold-change were obtained by relative quantification and $2^{-\Delta\Delta Ct}$ method (Livak KJ, Schmittgen TD, 2001). Analysis of relative gene expression data was done using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.), using DataAssist 2.0 Tool (Applied Biosystem, Foster City, CA, USA). Statistical analysis of miRNAs expression was performed using GraphPad Prism 5.0 tool.

3.2 MiRNA in OSCC cell lines

Two human oral tumor cell lines were analyzed: metastatic SCC2 cells and non-metastatic CAL27 cells. Cells were plated at $3x10^4$ cells/cm^2 in DMEM media supplemented with 10% fetal bovine serum (FBS). All human tumor cells were collected and cultured from the Department of Molecular and Cell Biology of Boston University, Boston, USA.
Using 15 Human Gene 2.0 ST arrays profiling human in OSCC SCC2 cells, with siRNAs-mediated knockdown of *DPAGT1*, we identified miRNAs with altered expression using a cut-off of -1.7 fold change (FC) and a p-value<0.05. All RNA used in the study was isolated using High Pure miRNA Isolation kit (Roche, Mannheim Germany). A NanoDrop ND-100 spectrophotometer was used to quantify and observe the quality of the isolated RNA. Total RNA was collected and purified using RNeasy mini prep kit (Qiagen). cDNA synthesis was performed using 1 µg RNA and iScript cDNA synthesis kit according to manufacturer's protocol. qPCR was performed using Fast SYBR green enzyme (Applied Biosystems) and measured on real time PCR system. Extracted miRNAs were quantified by qPCR to determine their relationship to changes in *DPAGT1* expression. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's t test.

Six miRNAs were selected for functional characterization in non-metastatic OSCC CAL27 cells transduced with lentiviral *DPAGT1* and metastatic SCC2 cells with lentiviral knockdown of *DPAGT1*. Lentiviral transduction of DPAGT1 in cell lines induces expression of mesenchymal markers, notably Zeb1, Twist1 and Twist2, thus they were studied in this study.

4. Results

4.1 MiRNA expression in OPML

The progressive OPML progressed to OSCC in the same site after a median period of 30 months, while the non-progressive OPML remained free of further recurrence after a median follow-up period of six years.

MiR191 and RNU44 were used as endogenous controls, as described previously, and they both revealed a good normalization score (1.6178) as indicated by DataAssist software. Of the miRNAs selected in this study, we have found a large number of miRNAs over-expressed and very few miRNAs showed a down-regulation. In normal oral mucosa miRNAs were not found over or under-expressed.

The following miRNAs passed the cut-off value of fold change:

- Non-progressive OPLM: miR-137, miR-181b, miR-196a, miR-375, miR-520g, miR-649;
- Progressive OPLM: miR-137, miR-146a, miR-181b, miR-196a, miR-345, miR-375, miR-518b, miR-520g, miR-649.

Significant results are related to miR-181b (p=0.0001) that exhibits an higher expression level in non-progressive OPML compared to the other groups; miR-649 (p=0.0057) shows

lower expression level in non-progressive OPML compared to progressive OPML (p=0.0334).

	Non-progressive OPML (FC)	**Non-progressive OPML (p-value)**	**Progressive OPML (FC)**	**Progressive OPML (p-value)**
Let7-d	1.184	0.7578	1.0649	0.859
miR-137	4.2758	0.1849	3.1068	0.0632
miR-146a	1.8982	0.4523	329.146	0.3458
miR-181b	***18.5864***	***0.0001***	12.7098	0.1118
miR-196a	97.5375	0.2995	75.2262	0.1113
miR-21	1.2084	0.6031	1.2095	0.6842
miR-221	1.8988	0.3118	1.4598	0.2986
miR-222	1.1502	0.7153	1.1944	0.5798
miR-345	0.6508	0.5492	222.926	0.3562
mir-375	4.2758	0.1849	10.0206	0.1024
miR-518b	1.8677	0.2651	409.851	0.3479
miR-520g	695.455	0.4615	193.086	0.2288
miR-649	***3.2471***	***0.0057***	***7.4628***	***0.0334***
miR-191	1.1973	0.5543	2.4957	0.2502

Considering differences between non-progressive and progressive OPMLs, the fold change expression of miR-146a, miR-345, miR-518b and miR-649 revealed a marked difference.

The marked over-expression of these miRNAs compared to normal controls and non-malignant oral lesions may indicate that the gradual genetic alterations, that lead a premalignant lesion to transform into cancer and metastasis, is mediated and regulated by

miRNAs. In particular miR-649 results the best candidate of the malignant progression of progressive OPML.

4.2 MiRNA expression in OSCC

The 26 cases of non-metastatic OSCC remained free of further recurrence and metastasis after a median follow-up period of 5 years.

Significant data were obtained for miR-21 (p=0.048) and miR-137 (p=0.048) between metastatic OSCC versus non-metastatic OSCC. In particular miR-21 was overexpressed while miR-137 resulted under-expressed.

	Fold Change	**P-value (<0.05)**
miR-21	2.594	0.048
miR-137	0.242	0.048

Regarding oral presurgical biopsies significant data were obtained for the overexpression of miR-21 (p=0.036) and under-expression of miR-10a (p=0.03).

	Fold Change	**P-value (<0.05)**
miR-21	4.22	0.0036
miR-10a	0.77	0.03

Significant data were obtained for miR-21 (p=0.048), miR-101 (p=0.005), miR-200a (p=0.019), miR-206 (p=0.013), miR-221 (p=0.042) and miR-345 (0.0006) between metastatic OSCC versus normal tissues. All of the miRNAs resulted over-expressed except for miR345 that was under-expressed.

	Fold Change	**P-value (<0.05)**
miR-21	4.38	0.005
miR-101	2.919	0.014
miR-200a	4.256	0.019
miR-206	152.35	0.013
miR-221	1.776	0.042
miR-345	0.297	0.0006

4.2 MiRNA expression in OSCC cell lines

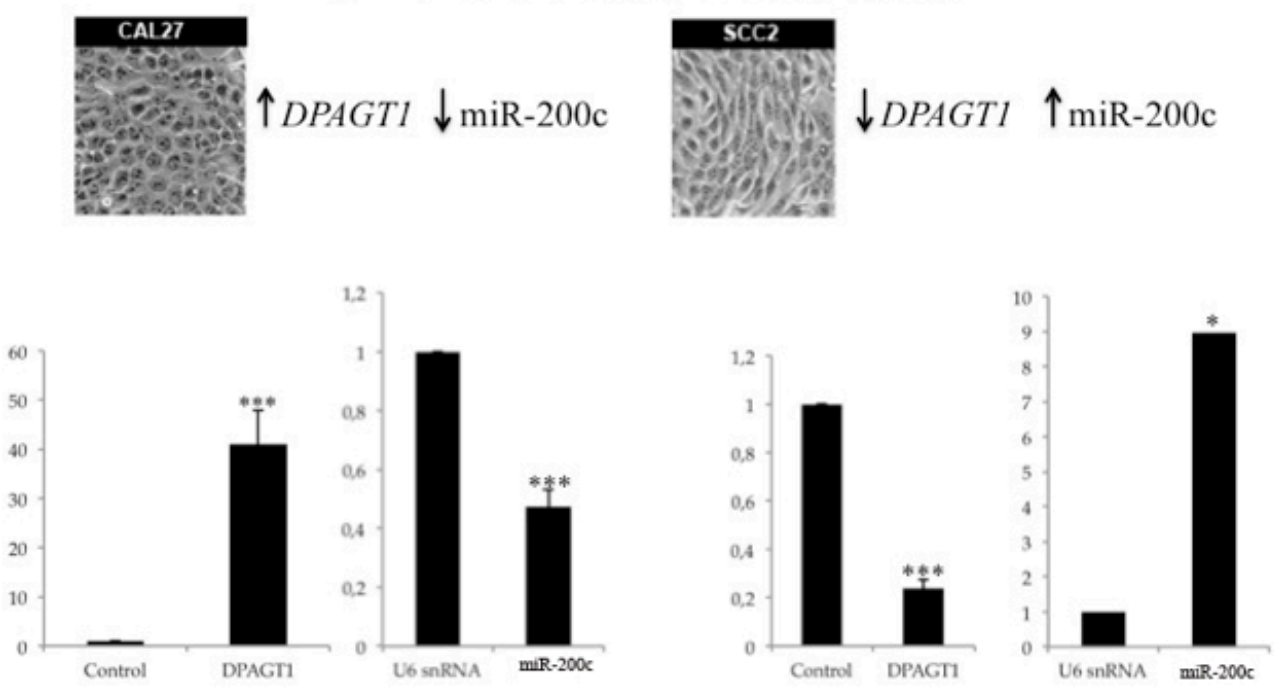
Twenty-five significant miRNAs related to *DPAGT1* knockdown were founded in the arrays. Approximately 1000 miRNAs were included in the array.

miRNA	Fold Change (cutoff - 1.7)	p-value (<0.05)
21	2.7	$6.7x10^{-10}$
23a	2.3	$6.3x10^{-5}$
24.1	2.5	$4.9x10^{-7}$
24.2	4.8	$1.2x10^{-8}$
27a	3.2	$1.4x10^{-7}$
31	2.5	$1.2x10^{-4}$
141	1.7	$1.9x10^{-2}$
181b-1	2.6	$1.2x10^{-3}$
200c	-2.1	$2.4x10^{-3}$
205	-2.8	$1.4x10^{-4}$
221	7.1	$3.4x10^{-7}$
222	2.3	$5.8x10^{-6}$
224	1.9	$4.3x10^{-2}$
503	-1.9	$4.3x10^{-4}$
570	1.7	$3.3x10^{-4}$
573	2.3	$3.7x10^{-3}$
590	2.3	$9.9x10^{-4}$
612	3	$1.5x10^{-4}$
711	1.7	$1.3x10^{-3}$
936	2.5	$2.2x10^{-6}$
1204	1.7	$4.8x10^{-3}$

3064	1.9	$5.8x10^{-5}$
3661	1.8	$7.2x10^{-4}$
3671	2.1	$7.6x10^{-3}$
5047	2.4	$1.7x10^{-5}$

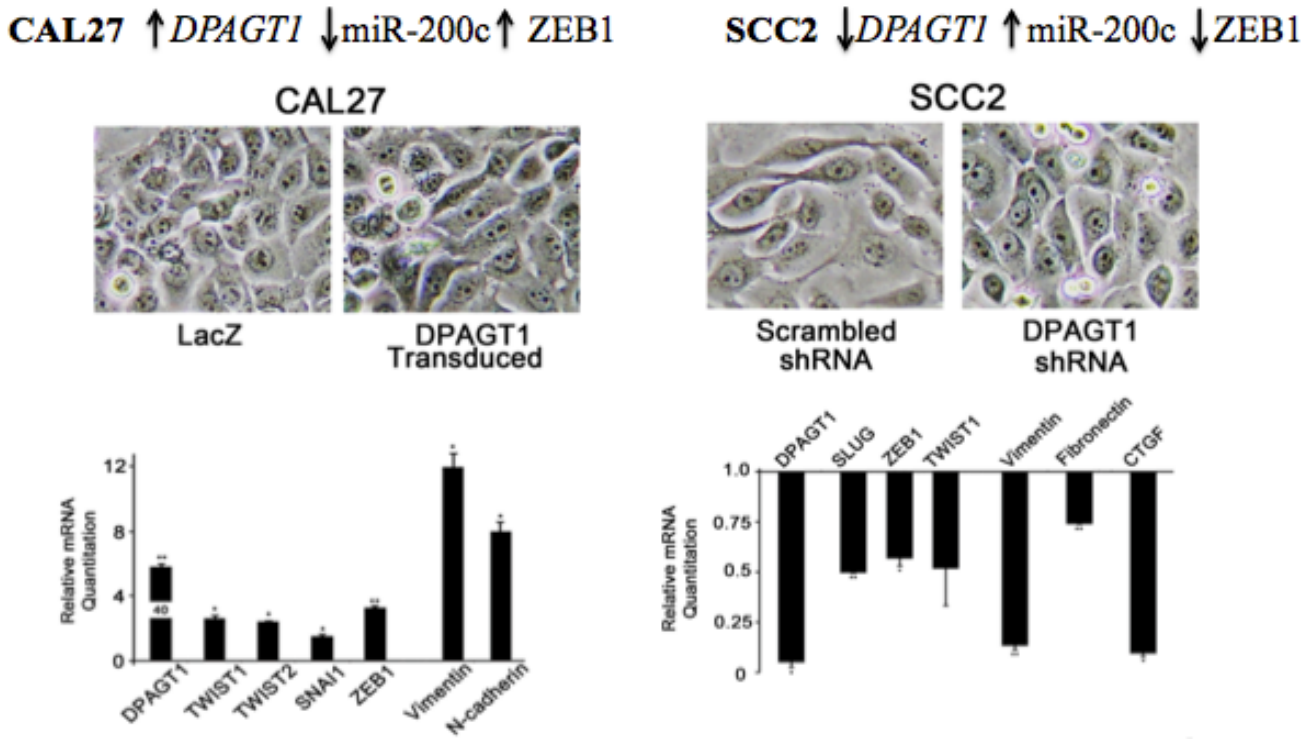
Of them, six miRNAs were selected based on their reported roles in EMT. They included: miR-21, miR-181b, miR-200c, miR-205, miR-221, miR-222. We extracted and measured their expression in CAL27 cells with induced expression of *DPAGT1* and in SSC2 cells with silenced expression of *DPAGT1*.

In SCC2 cells with *DPAGT1* knockdown, miR-200c was overexpressed (FC=11.5; p=0.04) while in CAL27 cells overexpressing *DPAGT1*, miR-200c was reduced (FC=0.47; p=0.01).

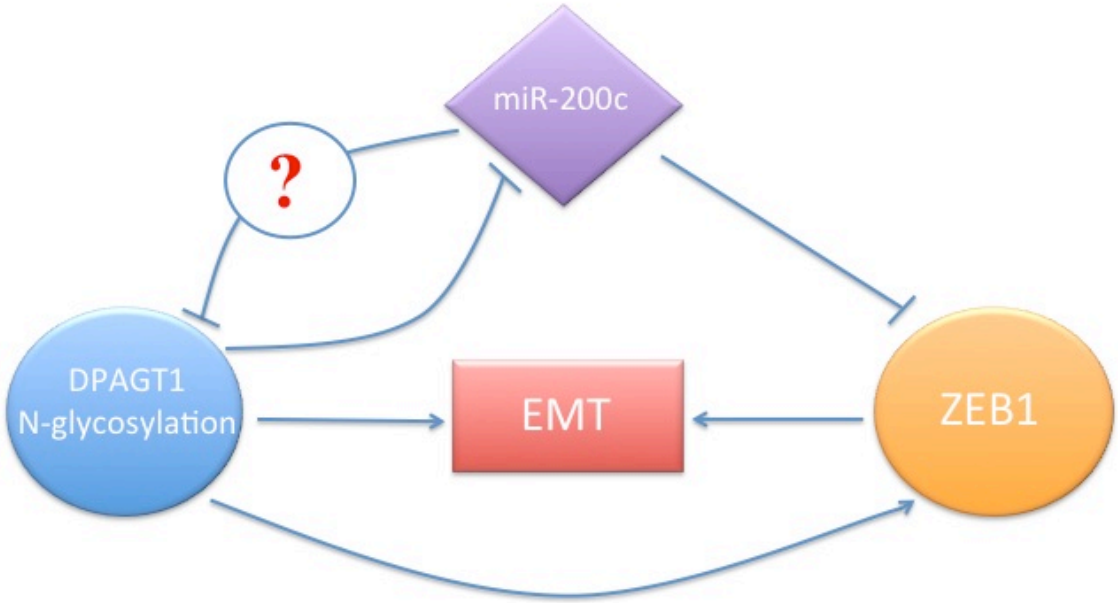


EMT-associated transcription factors, in particular ZEB1, expression were found down-regulated in SCC2 cells with *DPAGT1* knockdown and up-regulated in CAL27 cells transduced with *DPAGT1*. Moreover, when miR-200c was down-regulated, Vimentin and N-cadherin (markers of EMT) were highly expressed together with Twist 1-2 and ZEB1.

The microscope imagine shows the different form of the in vitro cells, in particular cells with *DPAGT1* overexpression are more elongated and detached each other, thus similar to mesenchymal cells, differently from the epithelial cells of the control. On the other hand, miR-200c overexpression resulted in down-regulation of EMT markers together with down-regulation of ZEB1 and Twist 1. Imagine from the microscope describe the reverse transformation from mesenchymal cells to cuboidal, attached cells like the epithelial cells.



The following figure described the algorithm between miR-200c, *DPAGT1* and ZEB1 related to the initiation of EMT. In particular, miR-200c acts as a tumor suppressor miRNA stopping ZEB1 in the initiation of EMT. The *DPAGT1* gene shows an oncogenic role by suppressing miR-200c and inducing EMT. Further studies need to reveal if miR-200c can inhibit *DPAGT1* gene directly.


miR-200c
?
DPAGT1
N-glycosylation
EMT
ZEB1

5. Discussion

Histological diagnosis cannot evaluate the risk of malignant transformation. Thus, miRNA has revealed to be good biomarkers for the management of these lesions. It seems that miRNAs can regulate important genetic steps that lead a dysplastic cell to transform into a cancer cell. In the present study, we explore the expression of miRNA in the malignant transformation of OPML and in aggressive OSCC cells and tumor samples.

MiRNA that revealed a diagnostic role and thus can be used as biomarker to differentiate non-progressive OPML to progressive OPML was miR-649.

MiR-649 was found over-expressed in OPML at high-risk of malignant transformation compared to non-progressive OPML. The marked over-expression of this miRNA compared to normal controls and non-malignant oral lesions may indicate that the gradual genetic alterations, that lead a premalignant lesion to transform into cancer is mediated and regulated by this miRNA.

In literature several miRNAs are described as over-expressed in OPML [47-49, 73, 74]. However, the most consistent study that describes a miRNA signature associated to progressive OPML is the study of Cervigne et al. [50]. They examined the miRNA expression in 29 leukoplakias that progressed to OSCC compared to 4 non-progressive leukoplakias and 7 normal tissues, and they found a large number of altered miRNAs. In particular, miR-21, 181b, 345, 146a, 518b, 520g, 649, 184 showed an increased expression during the progression to OSCC. The authors showed that some of the genetic alterations in OSCC are earlier expressed in the same-site leukoplakia.

Interestingly regarding miR-181b, Cervigne et al. reported an overexpression of this miRNA in progressive leukoplakias. However Yang et al. [48] revealed that miR-181b was found under-expressed in progressing leukoplakias compared to non-progressing. Our

data show a significant overexpression of miR-181b in non-progressive OPML in accordance with the article of Yang et al. These controversial data describe an uncertain role for miR-181b in OPML that need further studies.

In our results this miRNA showed a marked difference in terms of fold change between lesions that transformed into cancer and lesions that remained stable in a long-term follow-up period. Our study corroborates previous data reported by other authors [47, 50, 73] and seeks to underline the importance of introducing miRNAs in the all-day clinical practice of oral surgeons and pathologist in order to avoid the development of OSCC. MiRNAs represent important regulators of epigenetic expression and can be used for the early detection of OPML at high-risk of malignant transformation.

Regarding OSCC samples, we focused on T1 and T2 tumors because they present a better prognosis and nodal spread is usually confined to lymph nodes. Therefore the reason was to minimize the biological variations and to find biomarkers correlated to the early metastatic tumors. Thus, a diagnosis made by a miRNA biomarker at early stage or N1 stage can increase the survival expectation of the patient.

Our results showed a significant difference between miRNAs in T1 and T2 metastatic tumors and T1 and T2 tumors free of metastasis in more than 5 years. In particular, miR-21 was found overexpressed in aggressive OSCC that had metastasis in one or more cervical lymph nodes. MiR-21 is an established oncogenic miRNA that targets tumor-suppressing genes TPM1 and PTEN [55, 75]. Mir-21 was found over-expressed in squamous cell carcinoma of the tongue, and in progressive OPMLs [47, 48, 50]. This oncogenic miRNA promotes the tumor invasion of SCC of the tongue via the Wnt/β-catenin pathway by targeting tumor suppressor DKK2 [56]. MiR-21 was also involved in EMT in human bronchial epithelial cells and hepatocytes [58, 76].

Of interest, the miR-21 over-expression in presurgical biopsies revealed an important role of miRNA analysis directly prior to the surgery in order to make a correct diagnosis and orientate the prognosis. Mir-10a was found under-expressed in presurgical biopsies but not in OSCC samples. Very few studies are present in literature describing the role of miR-10a in oral cancer, thus further research is necessary.

On the other hand, we showed a down regulation of miR-137 in tumor samples, suggesting a tumor suppressor role in the events that lead to the metastasis as previously reported in literature [77]. Mir-137 seems to inhibit the mesenchymal biomarkers N-cadherin, vimentin and Snail expression indicating a suppressing role in EMT.

Regarding the miRNAs found in OSCC differently from normal oral tissues, we are not stupefied to see more overexpressed miRNAs because of their oncological role to promote OSCC. Mir-101 is described as a tumor suppressor miRNA, it is underexpressed in OSCC tissues and cell lines and inversely related to ZEB1 expression [78]. MiR-200a belongs to a different cluster of the miR-200 family (differently from miR-200c) but very few articles are described in literature about oral tissues, as well as about miR-345.

MiR-221 is known to be involved in tumorigenesis in several neoplasms, in particular in OSCC it is correlated to the growth of the tumor and p27 and p57 might be the targets of it [79].

Several studies reveal a miRNA expression in patients with metastasis of OSCC. Mir-29b, miR-155-5p miR-372, miR-373 are higher expressed in OSCC patients with lymph-node metastasis and thus they act as oncomirs in the malignant progression of OSCC [80-82]. Only two studies, similar to ours, reported a comparison between miRNA expression in patients with and without lymph node metastasis. In one article, the authors compared 20 metastatic OSCC with 17 non-metastatic OSCC and found 31 miRNAs differently

expressed, in particular miR-29a, miR-29c and miR-140-3p are involved in the downregulation of their specific target genes [83]. The other article reported an over-expression of miR-31 and miR-130b in non-metastatic samples, while miR-181 and miR-296 are over-expressed in metastatic tumors [84]. Regarding the discovery of miRNA in presurgical biopsies very rare oncological articles are reported in the literature. To best of our knowledge, only one article reported a miRNA expression in FFPE lymph nodes and fine-needle aspiration biopsies of OSCC patients. Mir-203 and miR-205 were found highly expressed in metastatic lymph nodes and showed high accuracy in fine-needle aspiration biopsies [85].

Our data suggest an important role of miR-21 both in oral presurgical biopsies and oral tissues as a prognostic factor in discriminating metastatic from non-metastatic OSCC. This result represents an important finding because no other studies describe a miRNA expression starting from biopsies and confirmed in tissues.

In OSCC cell lines, our results show that miR-200c is inversely related to *DPAGT1* expression and suggest that EMT and increased proliferation of complex N-glycans in OSCC are driven by changes by this miRNA. We identified ZEB1 as a predominant marker of EMT, in particular a significant correlation was observed between high ZEB1 expression and tumor cell proliferation associated with *DPGAT1* overexpression. We demonstrated that the inhibition of ZEB1 and *DPAGT1* in OSCC cell lines lead to significant inhibition of cell invasion in vitro guided by the overexpression f miR-200c.

The miR-200 family consists of five members, which form two clusters. MiR-200b, miR-200a and miR-429 are clustered on human chromosome 1, whereas miR-200c and miR-141 are grouped on chromosome 12, with each cluster expressed as a polycistronic transcript.

Binding specificities differ within the miR-200 family, with seed sequences differing between miR-200a-141 (subgroup I) and miR-200b-200c-429 (subgroup II) [86].
We have found that repression of ZEB1 by miR-200c resulted in reduced expression of the key mesenchymal markers, vimentin and fibronectin, and acquisition of an epithelial phenotype.
MiR-200 family members have subsequently been studied in a number of EMT-related in vitro model systems. During induction of EMT in MDCK cells with either TGF-b or ectopic expression of the protein tyrosine phosphatase Pez, the miR-200 family and E-cadherin were repressed in parallel with an increase in ZEB1 and ZEB2 expression [87]. The ability to induce an EMT was dependent upon repression of the miR-200 family and induction of ZEB1 and ZEB2 expression. Conversely, a MET could be induced by expression of the miR-200 family in cells that were originally mesenchymal in nature. These results confirm that the miR-200 family represses ZEB1 expression and consequently inhibits the progression of an EMT by establishing and maintaining an epithelial phenotype. The repression of ZEB expression by miRNA-200 family is direct, and occurs as a result of the miRNA binding to eight and nine sites in the 3 UTRs of ZEB1 and ZEB2 mRNA [69].
Data suggest that the majority, if not all, epithelial cells express high levels of the miR-200 family, which directly repress ZEB1 and ZEB2 and so enable the expression of E-cadherin. However, if an extracellular signal stimulates the expression of ZEB1, the miR-200 family is suppressed allowing EMT to proceed.

Together with ZEB1 expression we have found Twist1 expression. Twist1 is a highly conserved, basic helix-loop-helix transcription factor mapped at 7q21.2, has a bifunctional role, acting as an activator or a repressor, depending on post-translational modifications

and physiologic contexts [88, 89]. Twist1 induces gene transactivation through cisbinding to E-box regulatory regions, which are present in several target genes, and this involves complex homodimerization and heterodimerization mechanisms regulated by protein phosphorylation [89]. In the case of gene repression, Twist1 can repress genes by regulating chromatin remodeling through histone acetyltransferase-dependent=histone deacetylase-dependent mechanisms and through the inhibition of DNA binding activity of transcription factors [88]. The implication of Twist1 in cell migration is attributed primarily to its ability to contribute to EMT, through the down-regulation of E-cadherin and the upregulation of mesenchymal markers like vimentin, fibronectin, and N-cadherin [90, 91]. Previous studies have indicated that Twist1 promotes cell proliferation, migration, and expression of a primitive ECM, thus promoting an undifferentiated state [90]. In addition, Twist1 contributes to the EMT phenotype, which has been associated with resistance to chemotherapy and relapses [91].

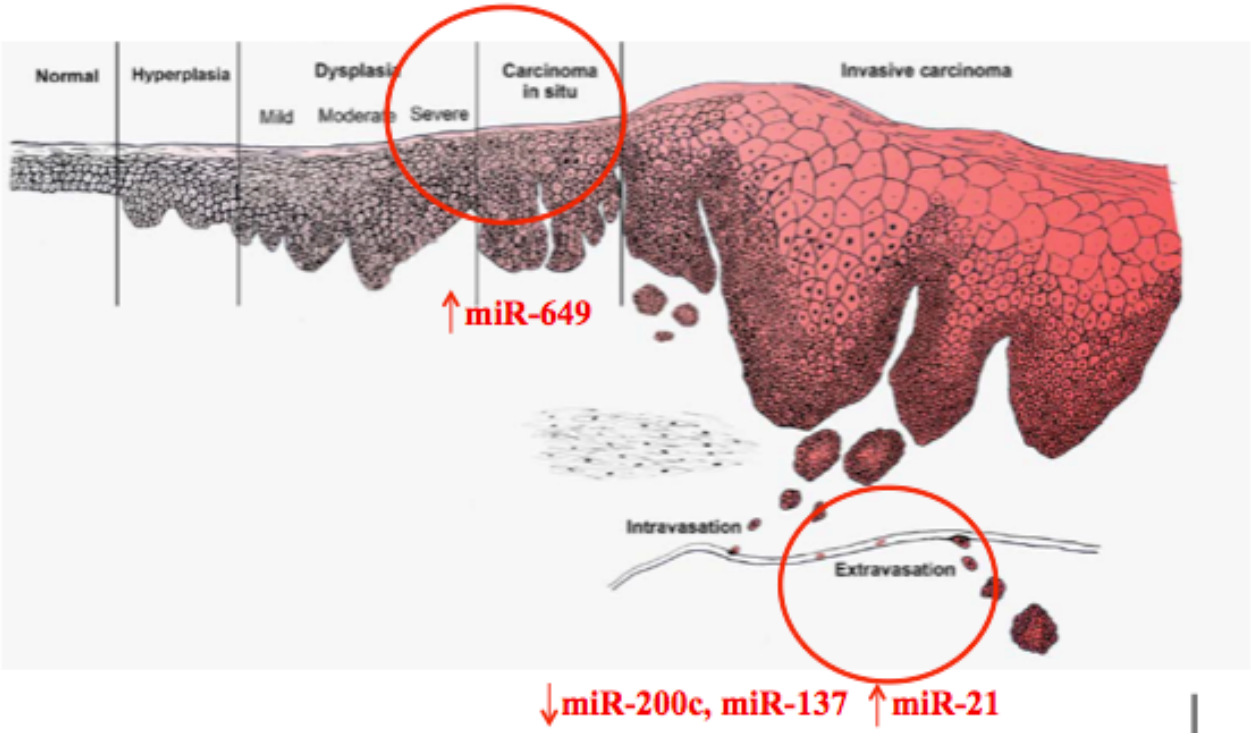
6. Conclusions

The progressive accumulation of genetic and epigenetic modifications leads the cell to undergo the neoplastic transformation. Thus, the use of molecules that regulate these processes are becoming important to prevent the genesis and growth of OSCC.

The overespression of miR-21 and downregulation of miR-137 may be used as prognostic biomarkers to differentiate metastatic OSCC between non-metastatic OSCC, while miR-649 can be used as a biomarker to prevent the malignant transformation of OPML. The miR-21 overexpression in presurgical biopsies of metastatic OSCC seems a useful biomarker to differentiate metastatic OSCC from non-metastatic OSCC.

In vitro data about tumor suppressor miR-200c must be tested in surgical samples and if good data will be obtained it may be used as a therapeutic option.

Our findings suggest that some miRNAs are correlated to an invasive behavior in OSCC and in OPML. The detection of these novel biomarkers can guide the surgeon to prevent the development of the tumor and lymph-node metastasis and to a better management of the patients.



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