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**PhD Thesis**

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# COMPUTATIONAL IDENTIFICATION OF EPIGENETIC ALTERATIONS AND ROLE OF THE TUMOR MICROENVIRONMENT AND ORAL MICROBIOTA IN ORAL SQUAMOUS CELL CARCINOMA DEVELOPMENT

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## ABSTRACT

The incidence of oral cavity cancer is widely increasing in the last years, mainly in Southeast Asian countries due to extensive use of tobacco and alcohol. Nevertheless, the incidence of this cancer seems to be enhanced also in young adults (age <40 years) in many Western countries. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity, representing more than 90% of all oral malignancies.

Although oral cavity is easily accessible to clinical examination, most of oral tumors are still diagnosed in an advanced stage (III and IV) and thus are associated with a high rate of mortality. Moreover, no reliable biomarkers are currently available for an early oral cancer detection. Thus, the identification of novel and effective biomarkers able to enhance both diagnostic and prognostic protocols in oral cancer is strongly necessary.

Recent decades have been characterized by great progress in understanding of the etiology, biology, and molecular basis of oral cancer and specifically, how the specific tumor microenvironment is able to interfere with cancer initiation and progression. Chronic inflammatory conditions, unbalanced signaling networks, alterations in microbiota composition, and epigenetic modifications contribute to establish a specific tumor microenvironment closely related to cancer initiation. Recently, microbiota alterations have aroused great interest due to their association with the development of human tumors, including oral cancer. Some specific bacterial strains such as *Capnocytophaga gingivalis*, *Fusobacterium spp.*, and *Streptococcus spp.* have been identified and considered to be able to contribute to carcinogenesis when deregulated.

Furthermore, the modified expression of specific genes and the consequent de-regulation of their signaling pathways has been clearly linked with carcinogenic degeneration. Interestingly, epigenetic modifications may assume a pivotal role in this scenario. The term "epigenetics" refers to heritable changes in gene expression that do not elicit modifications in the DNA sequence. Thus, DNA methylation, acetylation, histone modifications, chromatin remodeling, and noncoding RNA including microRNAs (miRs) mechanisms for gene silencing are implicated in oral cancer progression. miRs are small noncoding RNA, long 18/25 nucleotides which can affect gene expression, modifying the biological processes such as proliferation, apoptosis, and cell differentiation. Of note, they have been proposed as biomarkers of different stages of cancer, from early detection to prognosis.

Aim of this dissertation is to summarize the current evidence on oral cancer pathophysiology, and propose novel biomarkers in cancer diagnosis and prognosis. More in detail, in the **Chapter 1**, I present a general overview on oral cancer epidemiology, clinics and treatment, with a particular emphasis on recognized factor risks including tobacco, alcohol consumption, viral infections, preexisting oral lesions, and oral microbiota modifications. In regard to the oral dysbiosis, it is widely treated in the **Chapter 2**, focusing on mechanisms such as chronic inflammation, by which oral microbiota alterations could promote carcinogenesis. Then, the **Chapter 3** is devoted to the molecular pathways involved in oral cancer initiation, mainly to the role of miRs in cancer transformation and progression. Finally, the **Chapter 4** extensively deals with the experimental contribute performed to identify miRs with diagnostic and prognostic function in the OSCC. For this objective, GEO DataSets and TCGA Head and Neck Cancer (HNSC) databases have been analyzed. Moreover,

different computational approaches were used to detect the functional roles of these miRs. The analysis conducted has permitted to identify 11 miRs differentially expressed in tumor/normal samples and 8 linked with a different cancer stage progression. The set of miRs identified, after *in vitro* and *in vivo* validation, could represent a suitable tool for an early cancer detection and better understanding of its prognosis, supporting the clinicians in the best therapeutic choice.

# 1. EPIDEMIOLOGY, CLINICS AND TREATMENT: A GENERAL OVERVIEW

## 1.1 Introduction

Oral cancer (OC) represents the sixteenth common malignancy worldwide, principally occurring in individuals beyond 40 years with a variable incidence among different countries (Panta & Andreadis 2019). The most frequent histopathological type is the oral squamous cell carcinoma (OSCC), commonly localized on lateral border of the tongue, buccal mucosa, gingiva, and floor of the mouth (Stadler *et al.* 2008). Some recognized risk-factors such as tobacco (Chen *et al.* 2011), alcohol (Liu *et al.* 2015), viral infections (Gupta *et al.* 2013), nutritional deficiency (de Munter *et al*. 2015), poor hygiene habits (Holmes *et al.* 2009), chronic periodontal inflammation (Tezal *et al.* 2009), and mechanical trauma (Panta *et al.* 2018) have been connected with OCSS development. Moreover, precursor lesions and preexisting inflammatory conditions could promote the oral carcinogenesis (Piemonte *et al.* 2010). Recently, also a link between oral dysbiosis and cancer initiation has been established (La Rosa *et al.* 2020, Elebyary *et al.* 2021).

## 1.2 Epidemiological data

### *1.2.1 Incidence*

In accordance with the official estimates of the Global Cancer Observatory (GCO) of the International Agency for Research on Cancer (IARC) regarding the incidence, lip, oral cancer accounts for 377.713 new cases in 2020 and an age-standardized rate (ASR) of 4.1, with an increased trend compared to previous

years (GCO 2020). There are significant regional differences in new diagnoses: more than half of the new cases (i.e. 248.360) were recorded in Asia, followed by Europe (65.279) and Northern America (27.469) (**Figure 1.1**).

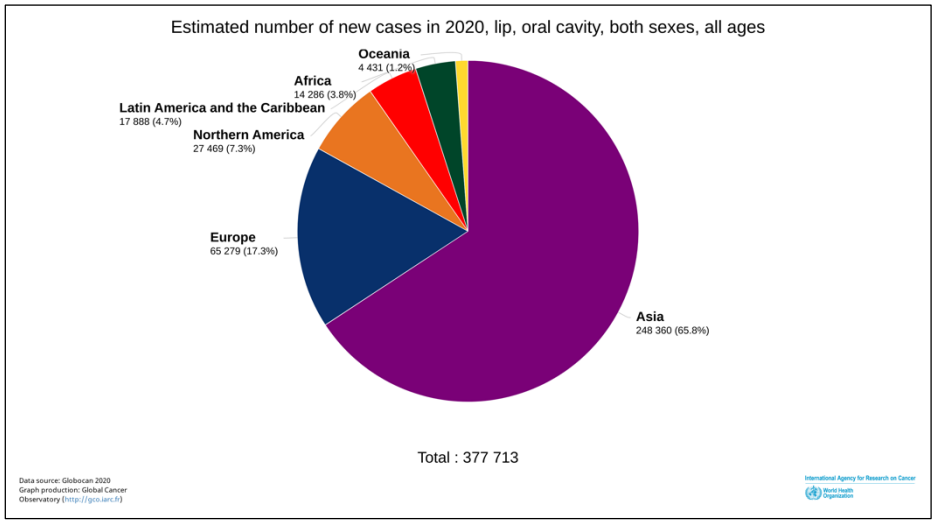


**Figure 1.1** Estimated number of new cases in 2020 regarding the lip,oral cavity, cancer (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

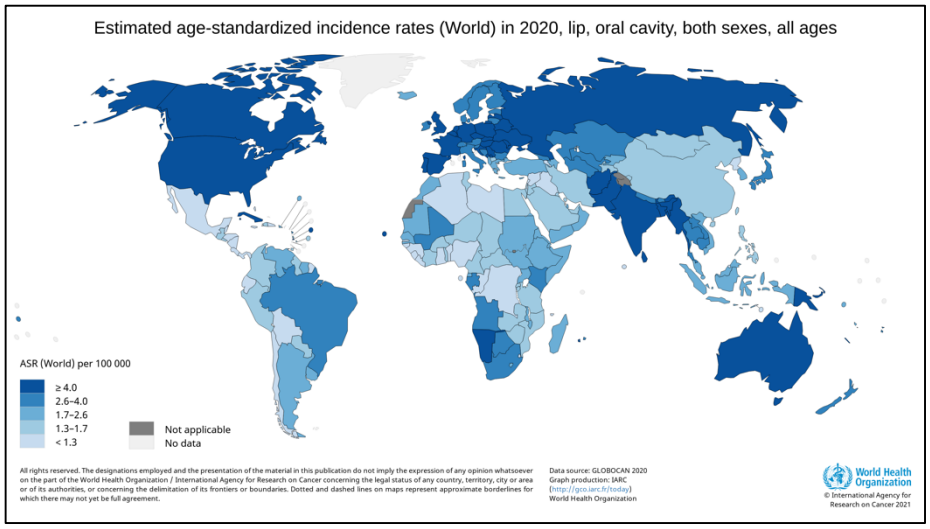


**Figure 1.2** Estimated age standardized incidence rates (World) in 2020 regarding the lip, oral cavity, cancer (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

India alone reported new 135.929 in 2020. Basing on the new registered cases, lip, oral cancer was the most frequent cancer among Indian males in 2020. The five countries with the highest ASRs in the world (Papua New Guinea, Pakistan, India,

Sri Lanka, and Bangladesh, in the order of frequency) were predominantly from the areas of the Oceania and Eastern and South Asia, confirming the previous trends (Bray *et al.* 2018, Ferlay *et al.* 2018) (**Figure 1.2**).

### *1.2.2 Favorite sites*

Favorite sites of oral cancer are variable and affected by the ethnicity, the country, the lifestyle including the smoking habits (i.e. chewing tobacco is associated with a major increase of buccal mucosa carcinogenesis). The most common sites include tongue, mandibular gingiva, maxillary gingiva, buccal mucosa, oral floor, and palate (**Figure 1.3**). Tongue cancer tends to occur at the lingual border, and it is uncommon to occur at the apex or back of tongue (Dhanuthai *et al.* 2018, Shrestha *et al.* 2020).

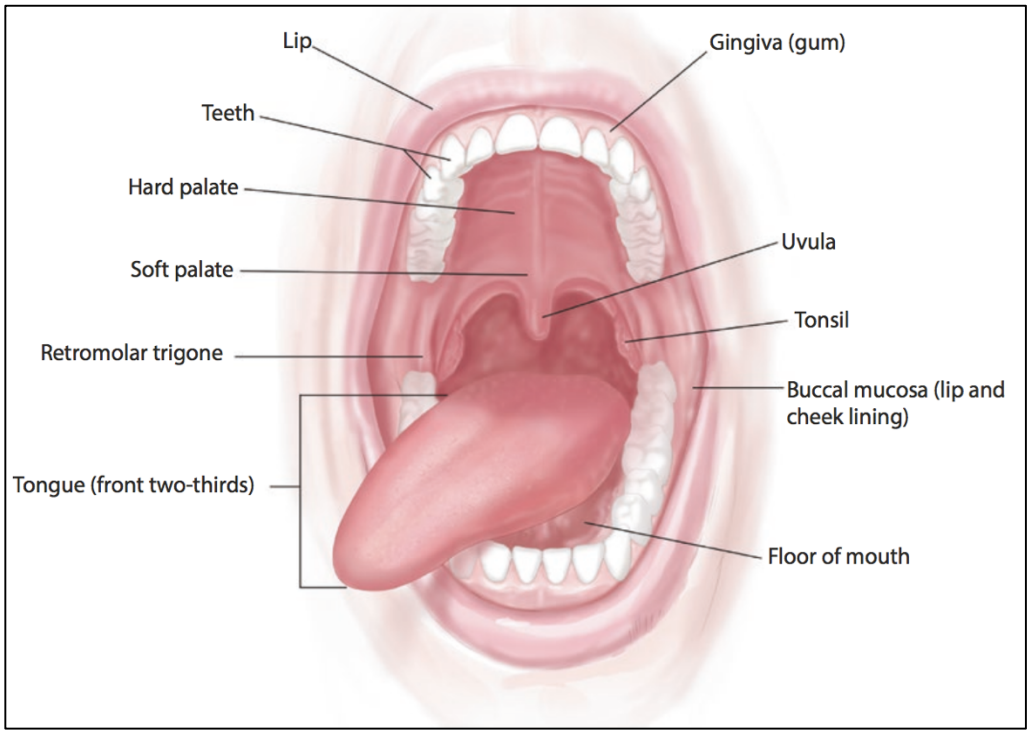


**Figure 1.3** Anatomy of oral cavity. The soft palate, uvula, and tonsil are parts of the oropharynx (*Source: Warnakulasuriya S, Greenspan JS. "Introduction-Cancers of the Mouth and Oropharynx" p.2 in Textbook of Oral Cancer, edited by Warnakulasuriya S, Greenspan JS, 2020. Springer International Publishing AG, Switzerland*).

### *1.2.3 Mortality*

Mortality rates widely vary according to geographical distribution, reaching up around 50% in many countries (Warnakulasuriya & Greenspan 2020) (**Figure 1.4**).

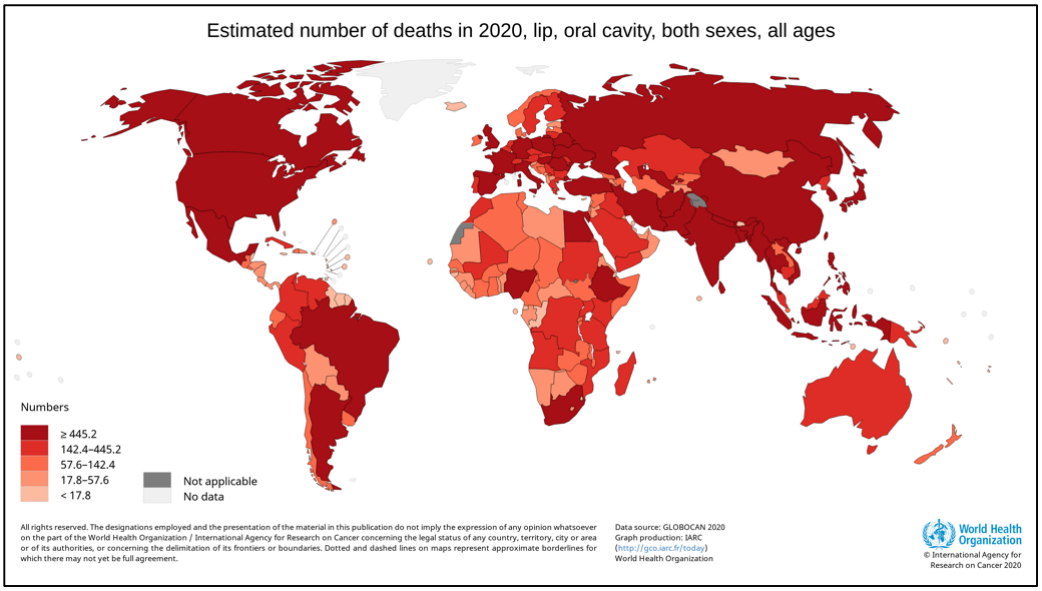


**Figure 1.4** Estimated numbers of deaths (World) in 2020 regarding the lip, oral cavity, cancer (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

According to the GCO data (GCO 2020), 177.757 deaths due to lip, oral cancer were registered in 2020 and 131.610 of these were localized in Asia. In the same year, the estimated deaths in males were above twice compared to females and mainly regarded the 60+ age group. The mortality rates have continued to increase in some Eastern European countries, such as Hungary and Slovakia, while some reduction was recorded in France and in the USA between 2002 and 2012, with an annual percentage reduction of −1% in some cancer centers (Warnakulasuriya & Greenspan 2020).

### *1.2.4 Survival*

The overall 5-year survival rate is around 85% for lip cancer. Nevertheless, considering oral cavity cancer, the rates are very variable depending on different factors, including tumor staging and also patient characteristics such as age and the presence of comorbidities. Late-stage disease, older age and comorbidities are negative prognostic factors for the survival rates (Yang & Warnakulasuriya 2016, Warnakulasuriya & Greenspan 2020). However, for patients with late-stage disease, 3-year survival rate for oral cancer has increased from 17.9% to 33.9% in the USA over the years 1998–2006, probably caused by the advances in the adjuvant chemoradiation and the existence of specialized cancer centers for the disease management (Schwam & Judson 2016). Likewise, an international study involving Germany, Italy, the USA, Brazil, India, Taiwan, and Australia showed an 11–12% rise in 5-year survival in the period 2001–2011 with regard to 1990–2000, attributable to an improvement in imaging and therapeutic tools (Amit *et al.* 2013). Despite advances in surgery and treatments, in many countries such as South Asia, the 5-year survival rates remain low, about or lower 50% (Sankaranarayanan *et al.* 2015).

### *1.2.5 Age and sex distribution*

The ASR estimated for oral cavity cancer in 2020 was 6.3 in men and 2.3 in women per 100.000, varying in different countries (GCO 2020). Comparing the incidence by age and sex groups, most the new cases has been reported in the 60+ group (i.e. 193.855) and males group (i.e. 264.211) (**Figures 1.5 and 1.6**). The gender differences in incidence have been linked with tobacco and alcohol habits which are higher in men, and the ratio is declining in geographical zones where women have assumed these habits (Warnakulasuriya & Greenspan 2020). A rising

incidence of oral cancer, especially tongue cancer, has been reported among younger people in several studies from many parts of the world (Patel *et al.* 2011, Monteiro *et al.* 2013, Shridhar *et al.* 2016a). Even if no strict definition of "young people" has been provided, most the studies reported 40 years old as a benchmark (Stimson & Guo-Pci 2002, Maruoka *et al.* 2005, Garavello *et al.* 2007).

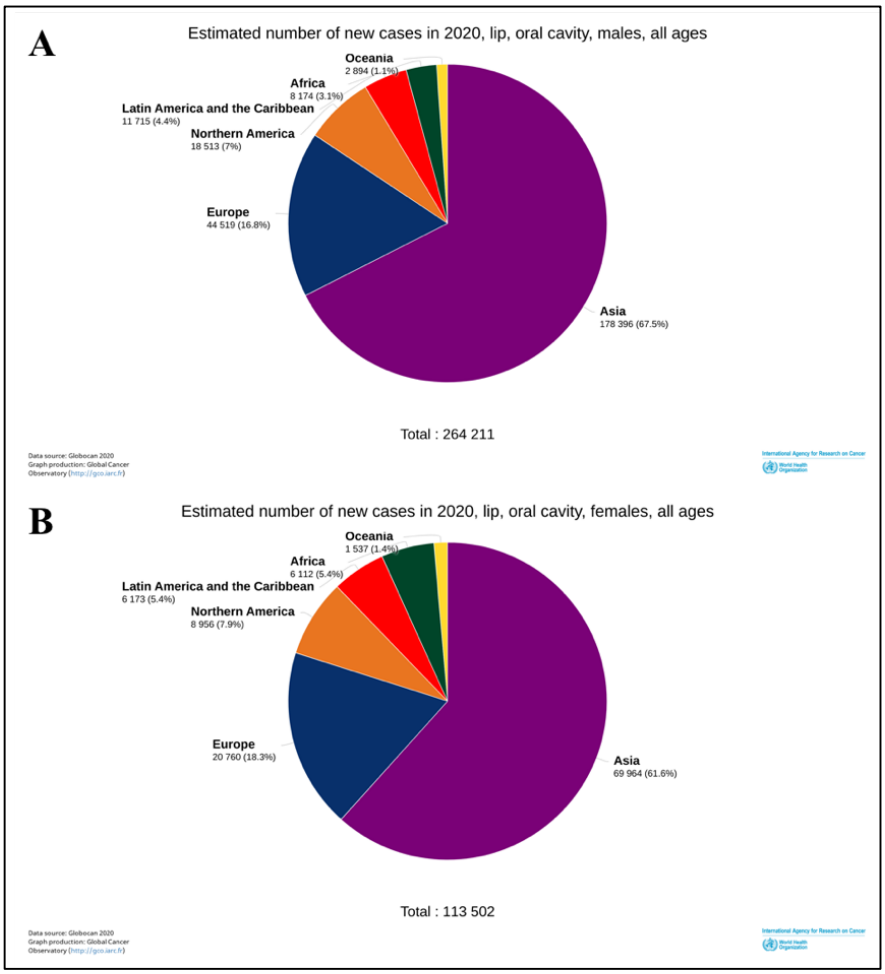


**Figure 1.5** Estimated numbers of new cases in 2020 regarding the lip, oral cavity, cancer in males **(A)** and females **(B)** (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

In accordance with a recent systematic review on oral cancer in young persons, the percentage of young cases from Australia (9.0%) and Europe (6.8%) were higher than reported from the USA (5.4%). The highest rates were registered in Africa (17.2%) followed by the Middle East (14.5%) and Asia (12.1%), likely

due to miscoding of lymphomas and odontogenic tumors of the oral cavity (Hussein *et al.* 2017).

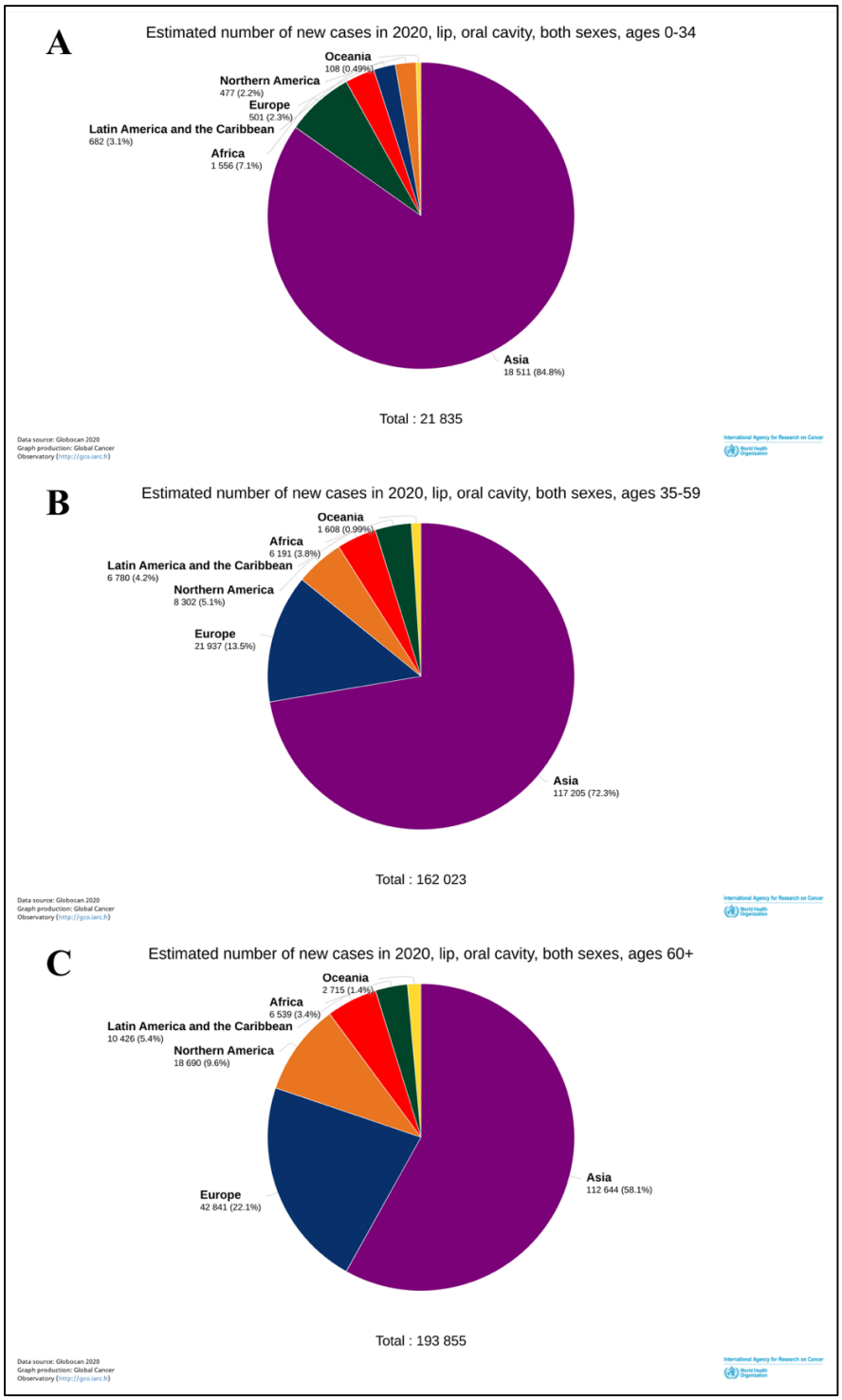


**Figure 1.6** Estimated numbers of new cases in 2020 regarding the lip, oral cavity, cancer in 0-34 **(A)**, 35-59 **(B)**, and 60+ **(C)** ages groups (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

Deaths rates increase with age, independently from sex and geographical distribution, registering the highest number in 60+ age group, as illustrated in the estimates updated to 2020 (**Figure 1.7**).

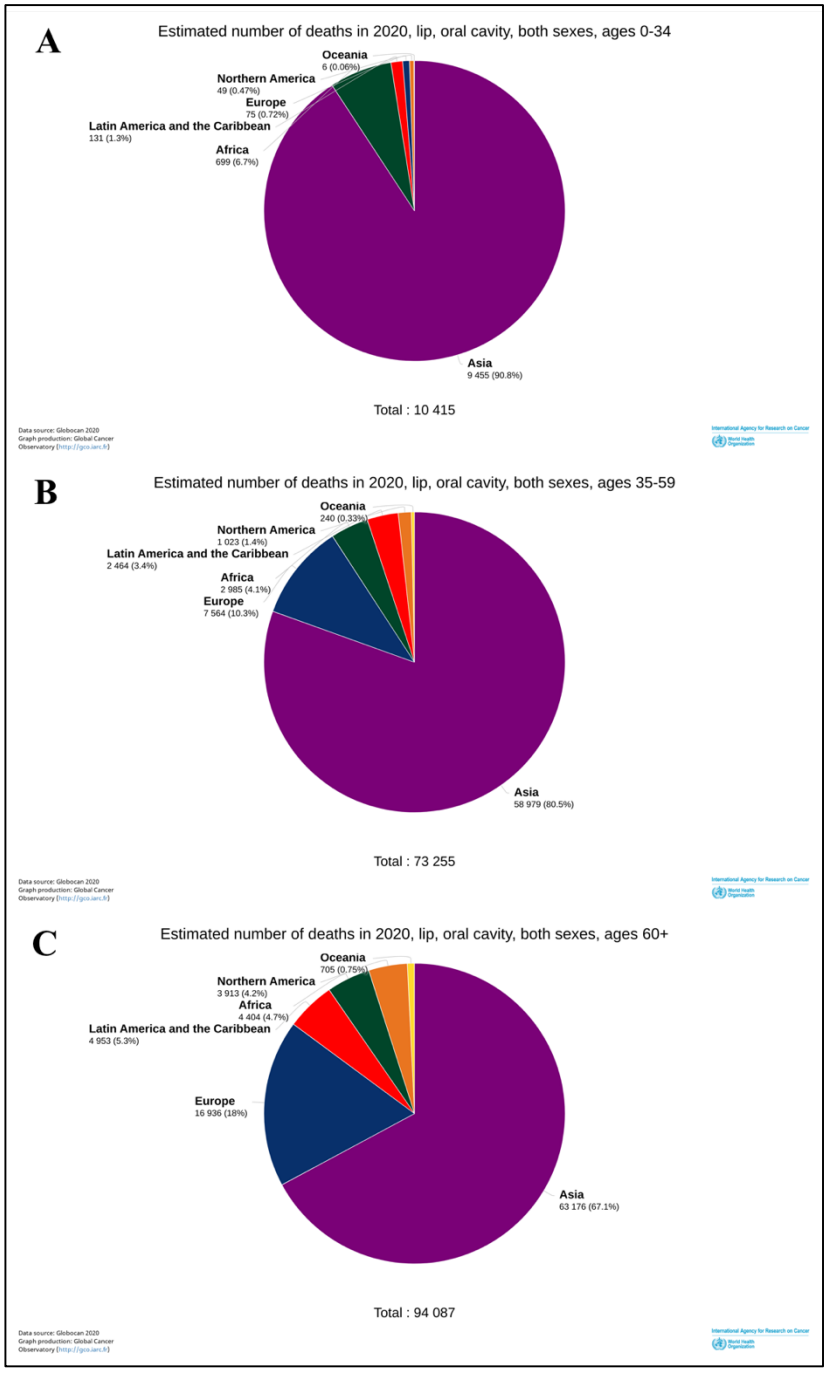


**Figure 1.7** Estimated numbers of deaths in 2020 regarding the lip, oral cavity, cancer in 0-34 **(A)**, 35-59 **(B)**, and 60+ **(C)** ages groups (*Source: GCO, Global Cancer Observatory: cancer today. Lyon, France: International Agency for Research on Cancer, 2020. Available from: https://gco.iarc.fr/).*

### *1.2.6 Socio economic conditions*

A certain association between oral cancer and socioeconomic status and deprivation has been reported, with the highest incidence rates shown in the poorest groups of the population. A systematic review of 41 publications confirmed that oral cancer risk is affected by social factors including education and employment (Conway *et al.* 2008). This association is particularly evident for men. An exception is represented by young population (under 45 years of age) in whom a quarter could be part of the professional classes (Warnakulasuriya & Greenspan 2020).

## 1.3 Risk factors

### *1.3.1 Tobacco*

Tobacco originates from two main species *Nicotiana tabacum* and *Nicotiana rustica*, which contain nicotine. Nicotine is a volatile alkaloid, known as one the most addictive and stimulant drugs. Even if nicotine effects regard all organs, it predominantly binds to a central nervous system receptor, enhancing brain dopamine levels (Dokhe *et al.* 2021). The mechanism of tobacco carcinogenesis is illustrated in **Figure 1.8**.

Tobacco is provided in numerous forms: smoking as cigarettes, cigars, pipes, bidis, or waterpipes; chewing as a smokeless tobacco product (SLT) which may contain other components such as betel quid or areca nut; or inhaling or sniffing through the nose as snuff (IARC 2012).

Tobacco in smoke phase includes many carcinogens substances like N-nitrosamines, polycyclic aromatic hydrocarbons, nitrosoproline, heavy metals, benzene, carbon monoxide and hydrogen cyanide (**Figure 1.9**).

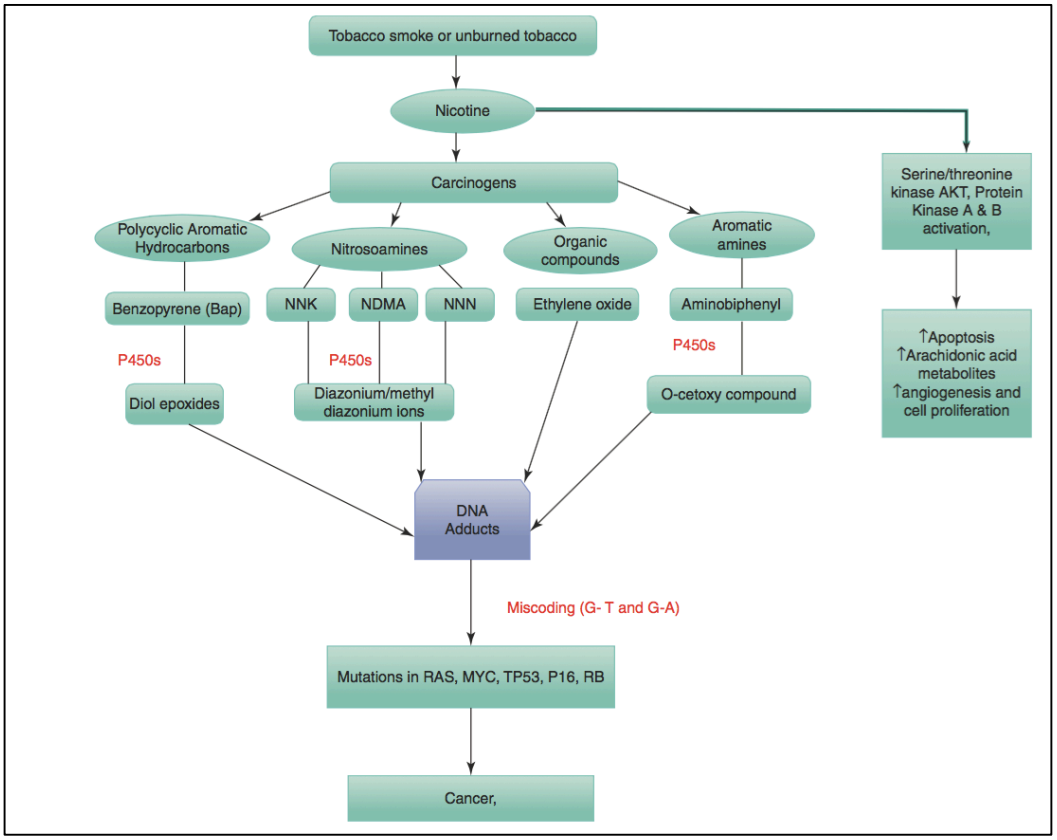


**Figure 1.8** Mechanism of tobacco carcinogenesis (*Source: Dokhe Y, Sivakumar V, Thankappan K, Iyer S. "Epidemiology", p.14 in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021. Springer International Publishing AG, Switzerland).*

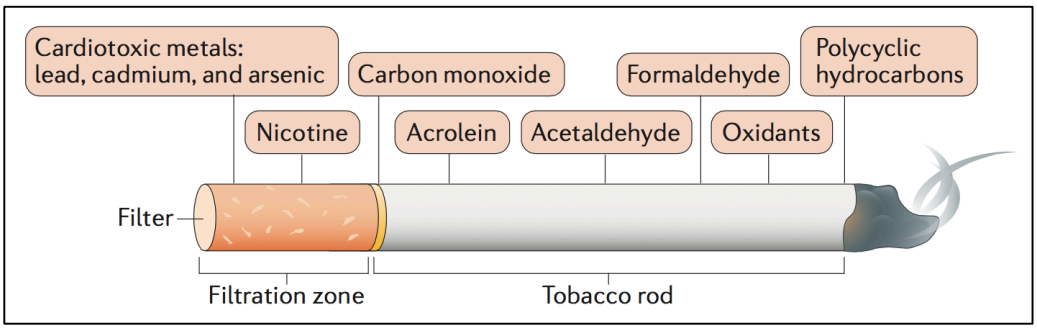


**Figure 1.9** Tobacco combustion substances contained in a traditional cigarette (*Source: Benowitz NL, Fraiman JB. Cardiovascular effects of electronic cigarettes. Nat Rev Cardiol 2017;14:p.449).*

Over 4000 chemical compounds have been detected, and about 62 of these have demonstrated sufficient carcinogenicity (Chen *et al.* 2011, Farsalinos *et al.* 2014). These compounds contribute to genetic alterations regarding several biological processes including oxidative metabolism, xenobiotic pathways, cell adhesion, and the mismatch repair system (Boyle *et al.* 2010).

Tobacco effects are mainly mediated through free radicals. In normal cells, the mutations are largely recognized and corrected by the mismatch repair (MMR) system. This system exhibits in smokers a covalent modification (i.e. hypermethylation) of their gene promoter that causes an expression loss, favoring the cancer transformation (Amaral-Silva *et al.* 2017). Tobacco smoking has been revised and included as a human carcinogen by the International Agency for Research on Cancer (IARC) monograph working groups in 1986, 2004, and 2012 (IARC 1986, 2004, 2012). Revisions conducted by IARC showed a direct relation for oral cancer risk between the number of cigarettes consumed per day and the tobacco smoking period. When people stop smoking, their oral cancer risk returns to normal value comparable to that of never smoking, after 10 or more years of quitting. Thus, a clearly causal association between oral cancer and tobacco smoking has been stated (IARC, 2004). As shown by an analysis of 13 case-control studies based on 4091 oral cancer cases and 18.664 normal, the risk due to cigarette smoking is about 2.87 (95% CI = 2.60, 3.18) (Wyss *et al.* 2013). Besides tobacco smoking, IARC has identified pipe and cigar smoking as strong risk factors for oral cancer development, with comparable magnitude of risk to cigarette smoking (IARC 1986, 2004).

Among other tobacco smoking forms, bidis deserve a particular mention. Bidis are locally tobacco products, especially consumed in South Asia, and are formed by coarse and uncured tobacco inside in tendu or *temburini* leaf, often filters free (IARC 2004, Hashibe 2020). Bidis have been recognized as carcinogenic in 2004 by IARC (IARC 2004) with a dose-response relation for oral cancer risk depending on duration and frequency (IARC 2012).

SLT products are numerous, widely spread amongst low-income and low-middle-income countries and are formed by more 30 carcinogens, such as tobacco-specific N-nitrosamines (TSNAs), nitrite, nitrate, heavy metals including nickel, cadmium, chromium, and arsenic. SLT is linked with OSCC, verrucous carcinoma, oral potentially malignant disorders (OPMDs) [i.e. leukoplakia, erythroplakia, and erythroleukoplakia, tobacco pouch lesion, and oral submucous fibrosis (OSF)] (Muthukrishnan & Warnakulasuriya 2018). SLT products include other elements such as betel quid (paan) or areca nut. Quid is constituted by betel leaf, areca nut, catechu, and slaked lime (calcium hydroxide) associated or not with flavoring agents. Areca nut present in betel quid exhibits carcinogenic effects attributable to arecoline and specific nitrosamines (**Figure 1.10**).

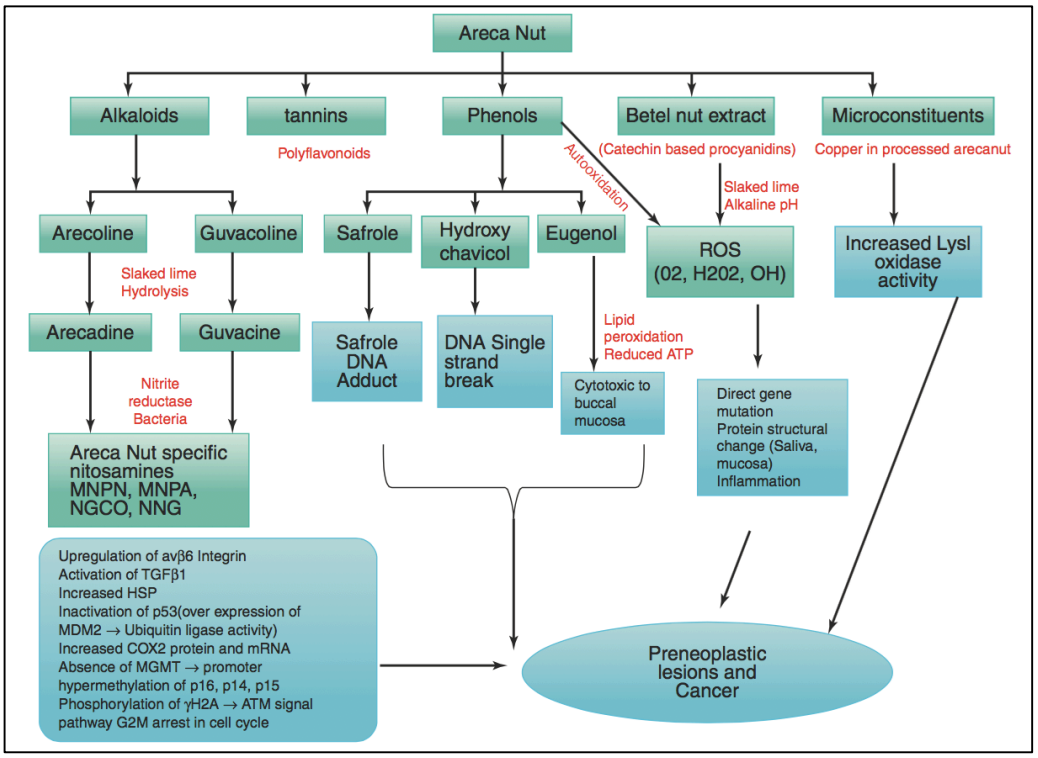


**Figure 1.10** Mechanism of areca nut carcinogenesis (*Source: Dokhe Y, Sivakumar V, Thankappan K, Iyer S. "Epidemiology", p.16 in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021. Springer International Publishing AG, Switzerland)*.

In addition, lime works as a tumor promoter by hydrolyzing alkaloids contained in the areca nut to cytotoxic and mutagenic compounds (Chaturvedi *et al.* 2013). Areca nut has been classified by some researchers as a psychoactive,

psychostimulant, and habit- or addiction-forming substance. Nitrosation of areco-line induces the formation of several compounds [i.e. N-Nitrosoguvacoline (NGCO), N-nitroso guvacine, 3 (Methylnitrosamino) propionitrile (MNPN), 3 (Methylnitrosamino) pro-pionaldehyde] which cause carcinogenesis (Shah 2012).

Betel quid habits are common in the East Asia (Taiwan, Mainland China, Malaysia, and Indonesia) and South Asia (Nepal and Sri Lanka). The betel nut chewing is more prevalent amongst men in Taiwan, Mainland China, Nepal, and Sri Lanka (Lee *et al.* 2015). Quid chewing represents an independent risk factor for oral cancer (Dokhe *et al.* 2021).

The use of electronic cigarettes (ECs) as an alternative to tobacco smoking has significantly increased in the last years (Polosa *et al.* 2019, O'Leary & Polosa 2020). ECs are battery powered electronic devices that causing the heating of a substance, generally a metal coil, vaporize a solution (e-liquid) basically formed by glycerol, propylene glycol (PG), distilled water, and flavorings, with or without nicotine. The consumer inhales the aerosol produced by vaporizing the e-liquid in a process known as ‘vaping.’ Many of the toxins present in cigarette smoke are also identified in EC aerosol, but at significantly less concentrations (Polosa *et al.* 2019). According to Yu et al. (2016a), e-cigarette vapor, both with and without nicotine, is cytotoxic to epithelial cell lines and is able to damage DNA. Nevertheless, cell-lines studies are not reliable simulation of clinical conditions in which e-cigarettes are used (i.e. frequency, exposure time) and may cause an overestimation or undervaluation of the effect (Yu *et al.* 2016a, Tellez *et al.* 2020, Vermehren *et al.* 2020) A human study involving 65 subjects (i.e smokers, e-cigarette smokers, and nonsmokers) concluded that electronic cigarettes appear to be safe for oral cells and should be suggested as tool to smoking cessation (Franco

*et al.* 2016). The rapid spread of e-cigarette use combined with the lack of prospective studies and with larger cohorts of participants justify the necessity for additional investigations to comprehend the oral health effects of these devices (Flach *et al.* 2019).

Finally, regarding the potential impact of involuntary smoking on oral cancer risk, the association is not established (Zhang *et al.* 1999, Lee *et al.* 2008, Lee *et al.* 2009, Troy *et al.* 2013, He *et al.* 2016). Further investigations are auspicious to address the hypothetical association between involuntary smoking and oral cancer risk.

### *1.3.2 Alcohol*

Alcoholic drinking refers to the consumption of alcoholic beverages including wine, beer, liquor, and other alcohol substances. Alcohol has been recognized as a carcinogen by IARC (IARC 1988, 2010, 2012). More specifically, ethanol is not carcinogenic but its metabolite acetaldehyde can jeopardize DNA repair (**Figure 1.11**).

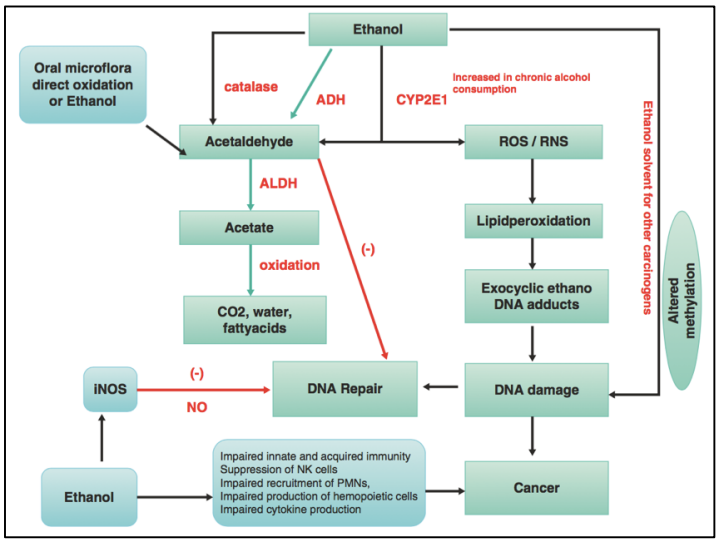


**Figure 1.11** Metabolism of alcohol and carcinogenesis-associated mechanisms (*Source: Dokhe Y, Sivakumar V, Thankappan K, Iyer S. "Epidemiology", p.17 in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021. Springer International Publishing AG, Switzerland).*

The cytotoxicity of ethanol depends on the exposure time and concentration as well as the ability to synergistically act with other carcinogens. Turati et al. (2010) evaluated the correlation of alcohol consumption with oral and neck cancers. The total relative risk (RR) of oral cancer for light drinkers (≤1 drink per day) in comparison with occasional or nondrinkers was 1.17 (CI, 1.01–1.35) while was significantly major in heavy drinkers (≥ 4 drinks per day) [RR 4.64 (95% CI, 3.78–5.70]. This trend was confirmed by another meta-analysis (Bagnardi *et al.* 2015) in which dose-response relations were evidently proved for alcohol drinking and the risk of oral/oropharyngeal cancers and were stable throughout experimental design, sex and geographic distribution. All studies provide convincing evidence that alcohol drinking has independent effect on the oral cancer risk, with robust dose-response association based on frequency and duration of alcohol drinking.

Alcohol drinking quitting reduces risk of oral cancer that returns similar to that of never drinkers after 10 or more years (Marron *et al.* 2010). All alcoholic beverages are correlated to a variable but still strong risk for oral cancer (Freedman *et al.* 2007, Marron *et al.* 2012).

### *1.3.3 Tobacco and alcohol association*

The synergic action of tobacco and alcohol consumption on oral cancer risk is well known. The major risk has been established for people who smoked >20 cigarettes per day and drank 3 or more alcoholic beverages per day (OR =15.49; 95% CI: 7.24, 33.14) (Hashibe *et al.* 2009). This reinforced effect on carcinogenesis is due to multiple mechanisms. First of all, alcohol injuries cell membrane phospholipids, enhancing their permeability, and consequently permitting the entrance of tobacco carcinogens. Moreover, high calorific value inhibits appetite, and thus reduces the benefits of food such as fruits and

vegetables. In addition, liver damage associated with alcohol can decreases the availability of enzymes involved in scavenging carcinogens (i.e. glutathione S-transferase and cytochrome p450). Finally, alcohol acts as good solvent for carcinogens and can catalyze the activation of pro-carcinogens and promote the DNA injury through its metabolites (Dokhe *et al.* 2021).

### *1.3.4 Viral infections*

An implication of certain viral agents such as human papillomavirus (HPV) has been proved in dysplastic and neoplastic transformations of squamous epithelium (Lynch 2006, La Rosa *et al.* 2021). HPVs are non-enveloped viruses presenting double-stranded DNA (Handisurya *et al.* 2009). More of 100 HPV subtypes have been recognized, with minimum 13 correlated with the occurrence of oral lesions (Ficarra *et al.* 1991, Miller *et al.* 2001, Hennessey *et al.* 2009). In particular, HPV-16 and−18 have been associated with dysplastic and neoplastic transformations of squamous epithelium (La Rosa *et al.* 2021). HPV is observed with interest as it constitutes the cause of oral cancer mainly in young people with no risk factors of smoking and drinking (Sasahira & Kuniyasu 2015). HPV life cycle and the processes by which HPV promotes carcinogenesis are illustrated in **Figure 1.12**.

Among the other viruses able to elicit head and neck carcinogenesis, Epstein Barr (EB) virus belonging to herpesvirus group (i.e. HSV-4) has been linked with Burkitt's lymphoma (Lynch 2006, Sasahira & Kuniyasu 2015).

Furthermore, oral mucosa can be affected by malignancies due to viral immunosuppression state induced by the human immunodeficiency virus (HIV). The most common malignant degeneration observed during HIV infection is

Kaposi's sarcoma (Curtiss *et al.* 2016, Cesarman *et al.* 2019).

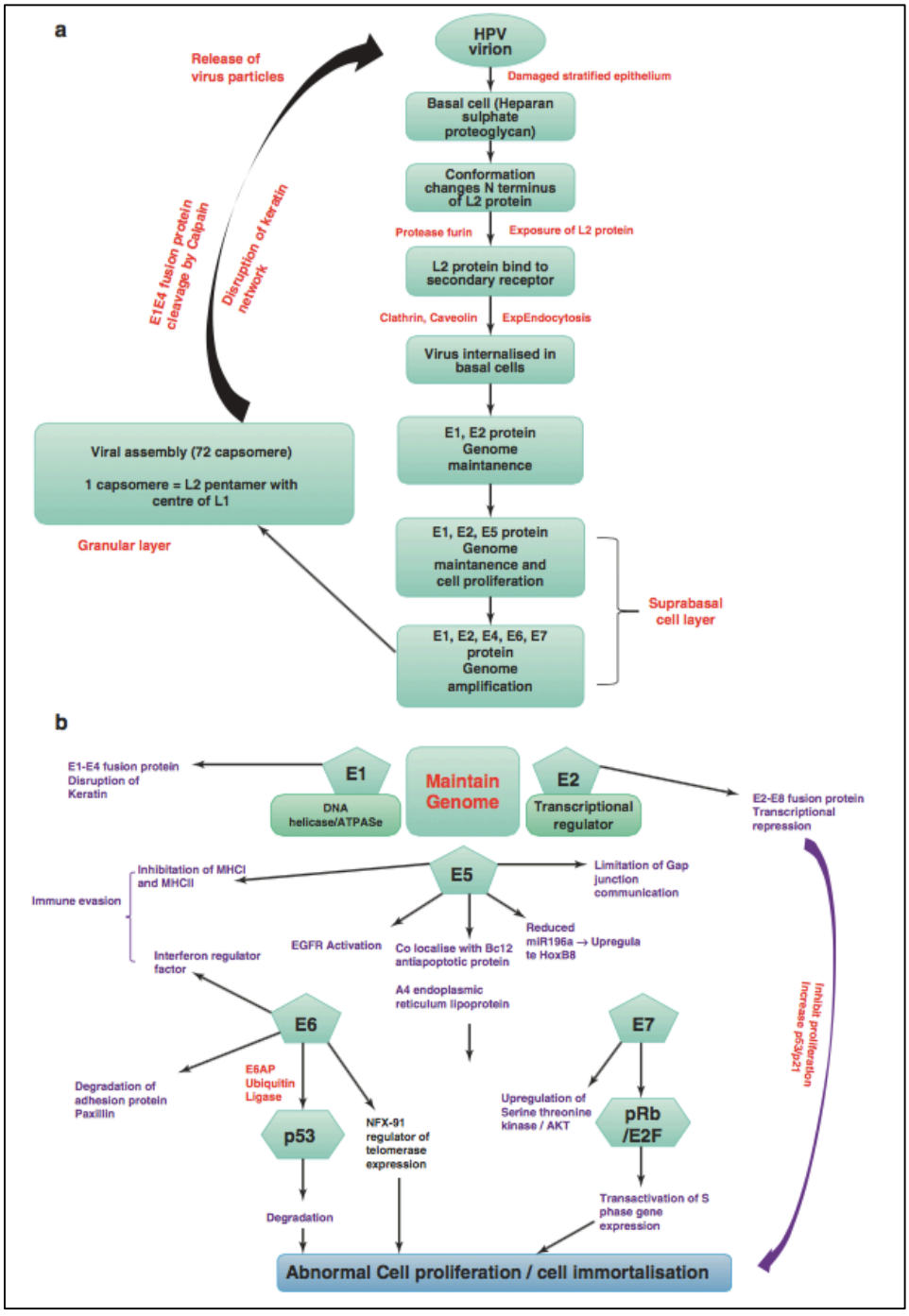


**Figure 1.12** HPV life cycle and carcinogenesis-correlated mechanisms (*Source: Dokhe Y, Sivakumar V, Thankappan K, Iyer S. "Epidemiology" p.18 in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021. Springer International Publishing AG, Switzerland)*.

### *1.3.5 Potential malignant disorders*

The WHO Collaborating Centre for Oral Cancer and Precancer in the UK recommended the term "Oral Potentially Malignant Disorders" in 2005 to replace the ambiguous terms "Oral Precancer" and "Oral Premalignancy" (Warnakulasuriya *et al.* 2007). Indeed, the new terminology emphasizes that not all disorders (also denoted in previous terminology such as "lesions" or "conditions") develop into oral cancer. OPMDs indicates a group of heterogeneous disorders involving oral mucosa, including leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis, lichen planus, actinic cheilitis, palatal keratosis associated with reverse smoking, discoid lupus erythematosus, dyskeratosis congenita, and epidermolysis bullosa (Warnakulasuriya *et al.* 2007). The risk of malignant transformation depends on numerous variables such as the type of OPMD, the characteristics of disorder such as color, location, size, the presence and grade of dysplasia as well as the kind of population examined (Mehanna *et al.* 2009, Arakeri *et al.* 2017, Yang *et al.* 2017).

Oral leukoplakia has been defined by the WHO working group 2005 as white plaques of questionable significance which cannot be identified as any other known disease (WHO 2005). It is a clinical term which includes hyperkeratosis of epithelia (hyperorthokeratosis, acanthosis, or hyperparakeratosis), with possible epithelial dysplasia, localized over the tongue, floor of mouth or gingiva, or gingivobuccal sulcus (Amagasa 2015, Bahadur *et al.* 2021) (**Figure 1.13**). The malignant transformation rate of oral leukoplakia is influenced by lesion features including nonhomogeneous pattern of lesions mainly those presenting a mix of red and white plaques (erythroleukoplakia), high degree of epithelial dysplasia, location, size and extension of lesion, lifestyle habits such smoking and alcohol consumption (Amagasa *et al.* 2006, Warnakulasuriya *et al.* 2007). Most

leukoplakias follow a benign course or regression if the risk factors are eliminated (Arduino *et al.* 2013, van der Waal 2014). A systematic review reported an average malignant transformation rate of 3.5% even if the reference range was wide (0.13% - 34.0%). The rate of annual transformation was between 0.3% and 6.9% (mean 3.8% per annum) with a follow-up period varying from 2.4 to 11 years (Warnakulasuriya & Ariyawardana 2016).

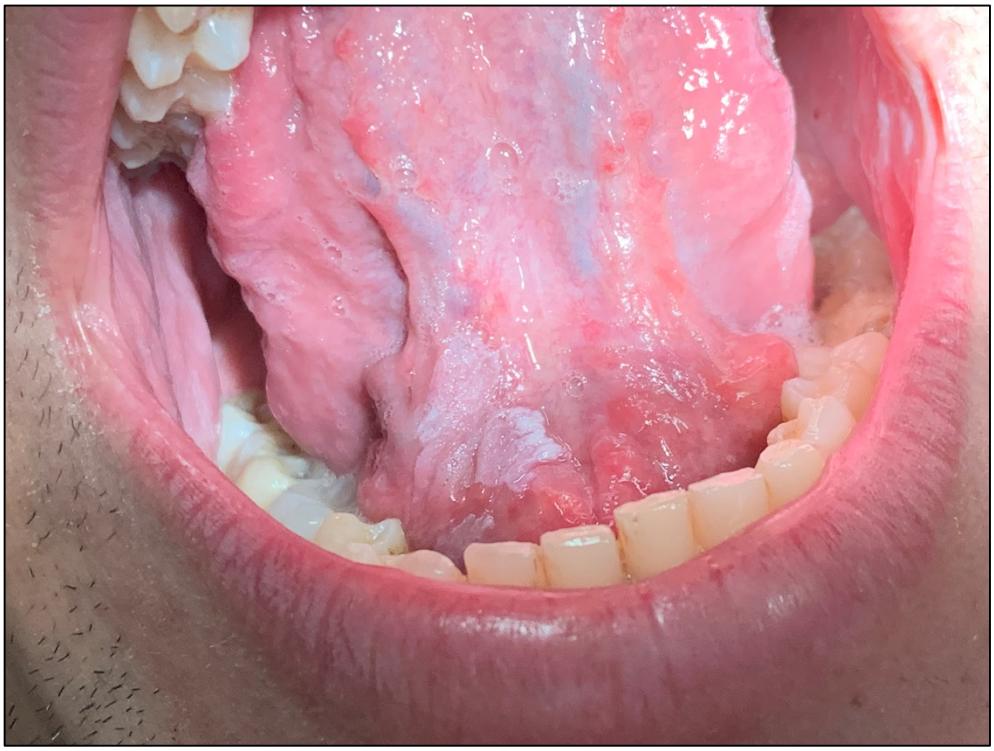

**Figure 1.13** Oral leukoplakia on the floor of the mouth *(Source: Personal archive).*

In accordance with the WHO definition, oral erythroplakia is "a fiery red patch that cannot be characterized clinically or pathologically as any other definable disease." Erythroplakia appears as a well-defined red lesion with flat macular velvety aspect that may be speckled with white spots (**Figure 1.14**). Most the clinically diagnosed erythroplakias show severe dysplasia which varies from carcinoma-in-situ (40%) to squamous cell carcinoma (51%) (Waal Vander & Axell 2002, Reibel 2003).

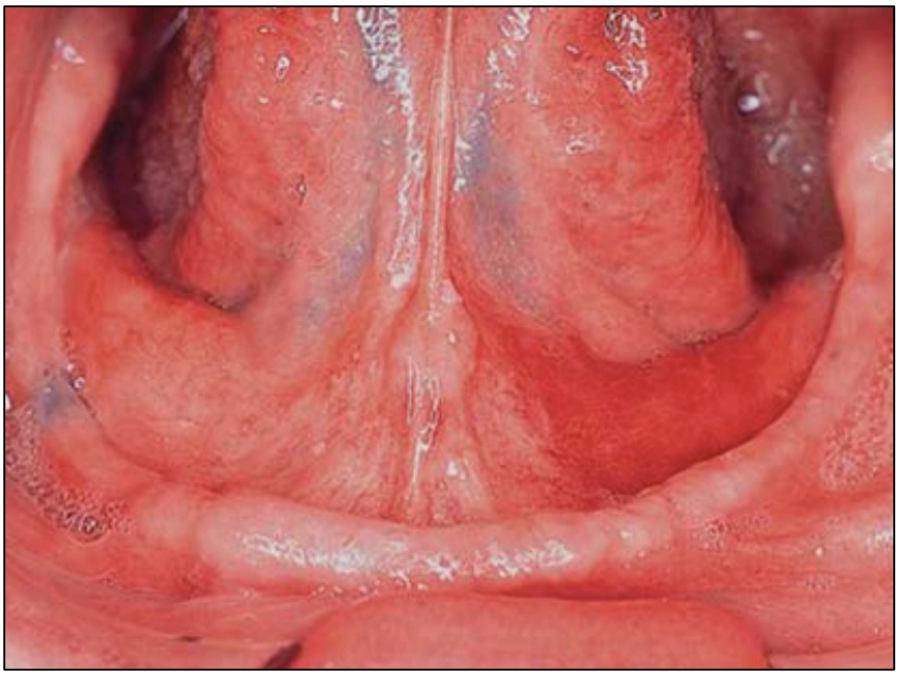

**Figure 1.14** Oral erythroplakia involving the left floor of the mouth *(Source: Giese RA, Boyle JO, Shah JP. "Management of potentially malignant disorders of the mouth and oropharynx", p.276 in Shah PJ & Johnson NW, Oral and Oropharyngeal Cancer, 2nd Edition, CRC Press, Taylor & Francis group, 2019).*

Oral submucous fibrosis (OSMF) is widely spread in the South-East Asia, often associated with nut consumption. Its malignant range varies from 0.5 to 6%. Interestingly, among risk factors, genetic factors and immunologic predisposition could contribute to the disease development. Moreover, as reported by Ranganathan *et al.* (2001), a significant decrease in plasma cholesterol levels and serum beta-carotene levels has been noticed in patients affected by OSMF. The clinical presentation is marked by a progressive reduction in mouth opening due to fibrosis of lamina propria and submucosa with consequent loss of tissue mobility. Other clinical manifestations include burning sensation on the ingestion of spicy foods, vesicles and ulcers, blanched mucosa, and reduced jaw movements (Bahadur *et al.* 2021) (**Figure 1.15**).

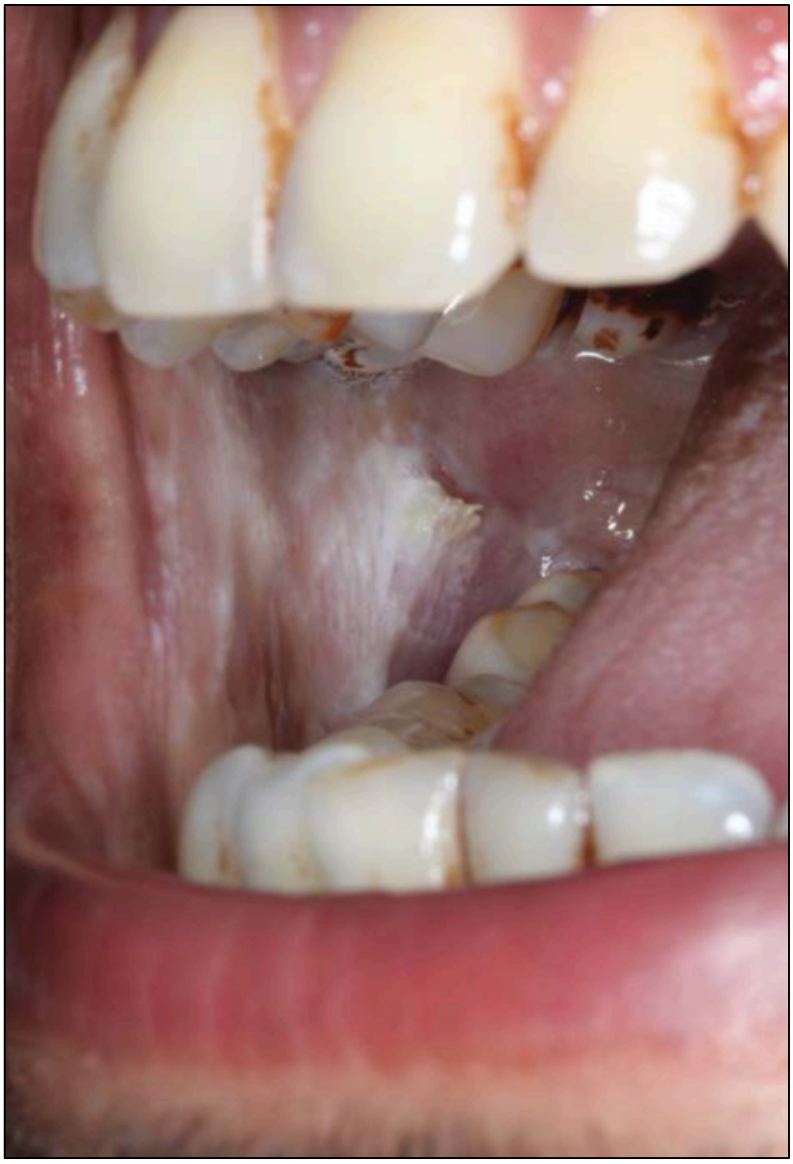

**Figure 1.15** Oral submucous fibrosis with hyperkeratosis of the buccal mucosa and limited opening of the mouth *(Source: Farah CS, Jessri M, John K, et al. "Clinical features and diagnosis", p.106 in Oral and Oropharyngeal Cancer edited by Shah PJ & Johnson NW, 2nd Edition, CRC Press, Taylor & Francis group, 2019).*

Another common oral potential malignant disorder is lichen planus. It is a chronic inflammatory condition showing some degree of immune pathology, in which T lymphocytes accumulate beneath the epithelium of oral mucosa, resulting in hyperkeratosis and erythema with or without ulceration (Epstein *et al.* 2003). Several clinical sub-types have been distinguished and the symmetrical, reticular form is the most recurrent clinically described (Warnakulasuriya 2020) (**Figure 1.16**).

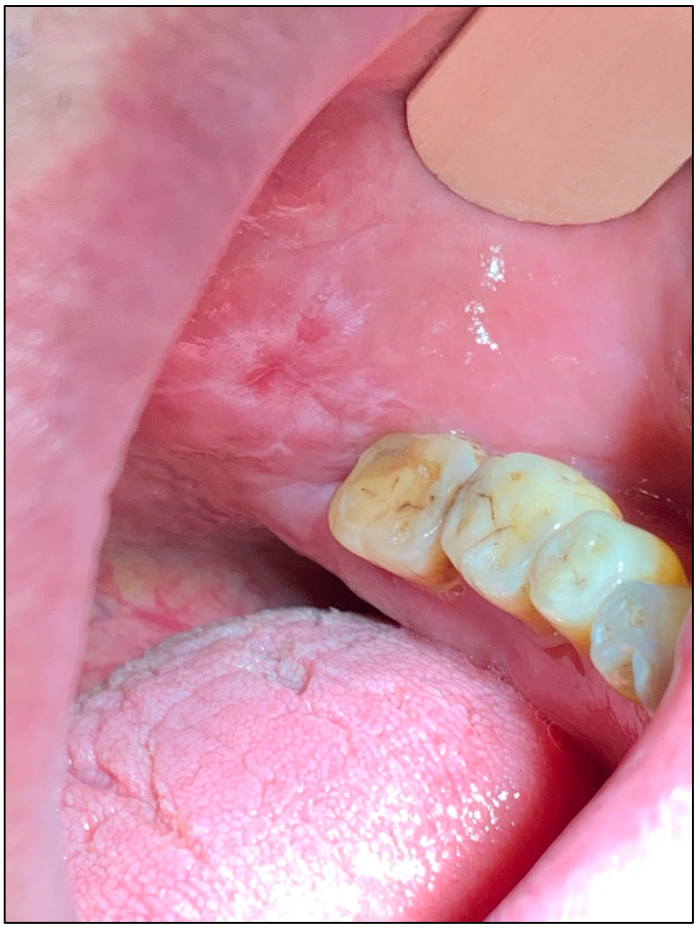

**Figure 1.16** Reticular form of oral lichen planus, in the buccal mucosa *(Source: Personal archive).*

Actinic cheilitis (AC) is a chronic inflammatory condition which clinically appears as keratosis with crusting, flaking, and dry skin and sometimes with ulcerations (Jadotte & Schwartz, 2012). (**Figure 1.17**). Excessive exposure of lips to solar ultraviolet (UV) radiation is a well-known risk factor for AC and for lip cancer development (Wood *et al.* 2011, de Oliveira *et al.* 2014). Protection from direct solar radiation reduces this risk (Hashibe 2020). The current available evidence on the malignant degeneration rate of AC is limited. A recent systematic review of the current literature reported a malignant degeneration rate of 3.07% basing on a single research article (Dancyger *et al.* 2018).

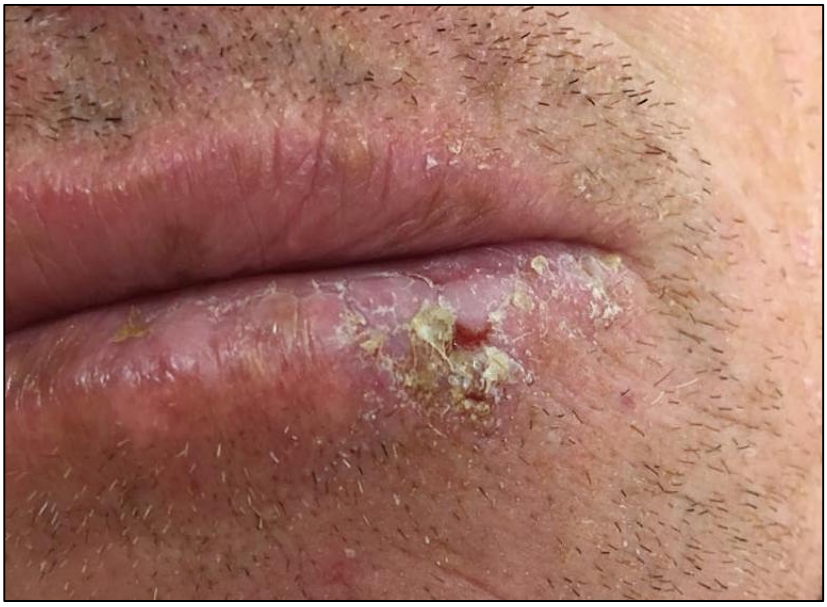

**Figure 1.17** Actinic cheilitis involving the lower lip *(Source: Personal archive).*

Palatal changes of reverse smokers include "thickened leukoplakic plaques, mucosal nodularity, excrescences around orifices of minor mucosal glands, yellowish brown staining, erythema and ulceration" (Gupta *et al.* 1980) (**Figure 1.18**). Palatal changes in reverse smokers exhibit a higher hazard ratio of malignant transformation in comparison with leukoplakia (Warnakulasuriya 2020).

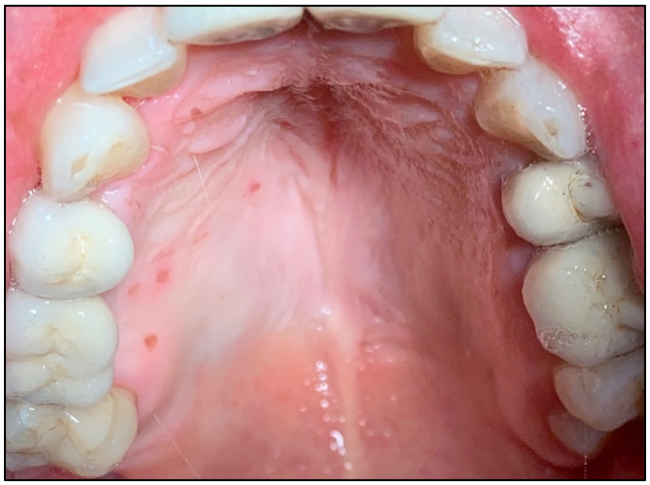

**Figure 1.18** Palatal changes of reverse smokers with evidence of yellowish brown staining *(Source: Personal archive).*

Discoid lupus erythematosus (DLE) is a rare autoimmune disorder which is limited to skin and mucous membrane. About 20% of DLE patients present oral manifestations principally affecting palate, buccal mucosa, tongue, and vermilion border of the lip (Liu *et al.* 2011) (**Figure 1.19**).

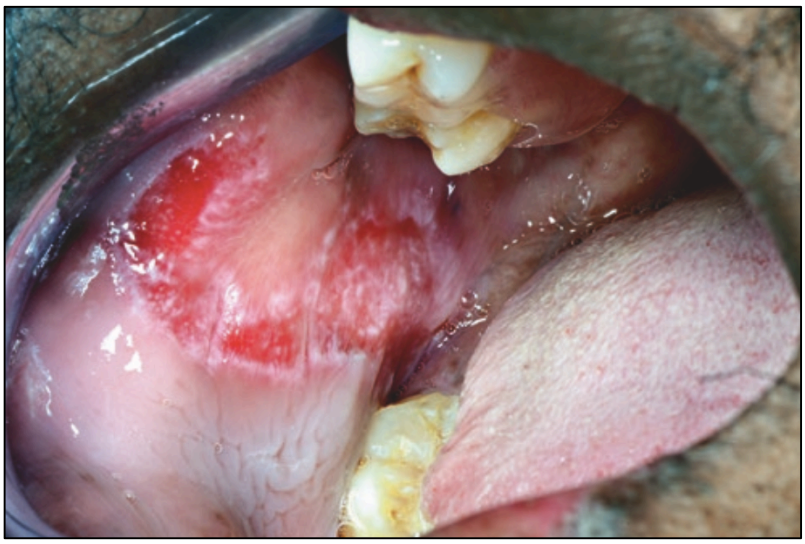

**Figure 1.19** Oral lesions on the buccal mucosa occurring in discoid lupus erythematosus disorder *(Source: Warnakulasuriya S. "Potentially Malignant Disorders of the Oral Cavity" p.148 in Textbook of Oral Cancer, edited by Warnakulasuriya S, Greenspan JS, 2020. Springer International Publishing AG, Switzerland).*

Dyskeratosis congenita (DC) is another rare inherited syndromic condition connected with a bone marrow failure and a growing risk of oral malignancy. Oral leukoplakia is diagnosed in 65–80% of patients affected by DC (Bongiorno *et al.* 2017). The typical triad of clinical manifestations includes leukoplakia of the dorsal tongue (Handley & Ogden 2006), reticular hyperpigmentation of the skin, and dystrophic nails (**Figure 1.20**). Leukoplakic patches in DC patients have shown malignant transformation rate with a high frequency. DC is caused primarily by specific mutations of the DKC1 gene encoding for dyskerin involved in the maintaining of telomeres (Abdel-Karim *et al.* 2009).

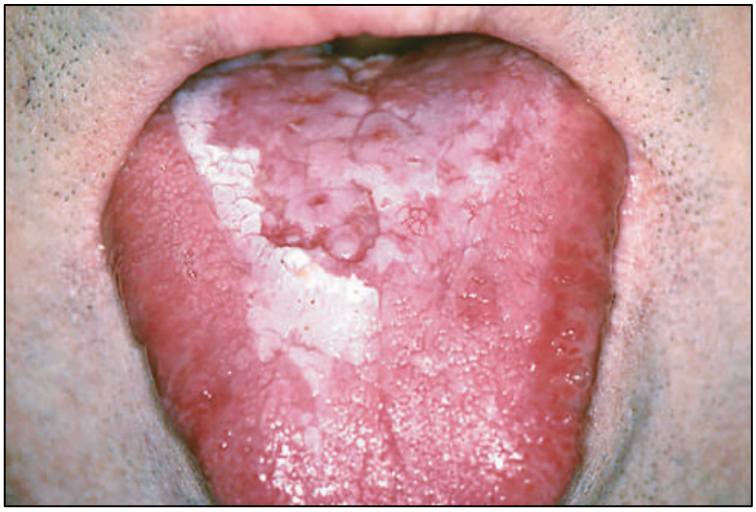

**Figure 1.20** Leukoplakia of the dorsal tongue in a patient affected by dyskeratosis congenita (*Source: Color Atlas of Oral Diseases. Diagnosis and Treatment edited by Laskaris G, 4th ed. New York, NY: Thieme; p.309, 2017*).

Epidermolysis bullosa (EB) is a blistering skin disease with an underlying epithelial fragility confirmed by the vesicular-bullous eruptions and superficial erosions of the oral mucosa. (**Figure 1.21**). Specific oral premalignant lesions associated with EB are not well documented. Chronic ulceration is likely linked with an increased risk of oral squamous cell carcinoma in EB (Warnakulasuriya 2020).

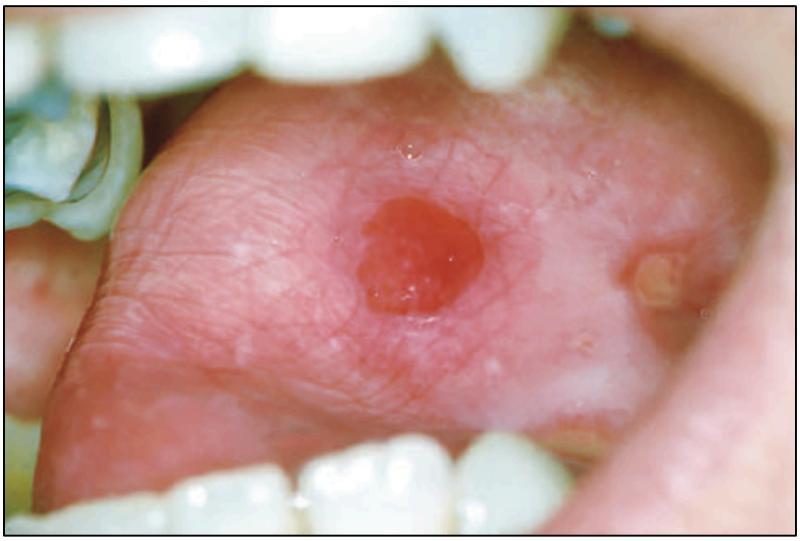

**Figure 1.21** Vesicular-bullous eruption on the tongue in patient affected by epidermolysis bullosa (*Source: Color Atlas of Oral Diseases. Diagnosis and Treatment, edited by Laskaris G, 4th ed. New York, NY: Thieme; p. 324, 2017*).

### *1.3.6 Oral dysbiosis*

Microbiota modifications have been connected with the initiation and progression of human cancers. Precise bacterial strains have been identified and strongly linked with oral cancer development (i.e. *Capnocytophaga gingivalis*, *Fusobacterium spp.*, *Streptococcus spp.*, *Peptostreptococcus spp.*, *Porphyromonas gingivalis* and *Prevotella spp.*). Oral dysbiosis promotes cancer pathogenesis by means of different mechanisms including chronic inflammation, microbial synthesis of cancerogenic substances, and alteration of epithelial barrier integrity. This topic is covered in detail in the following chapter.

## 1.4 Clinical features

### *1.4.1 Early stage*

Oral cancer exhibits multiple clinical manifestations, which sometimes are difficult to diagnose mainly during the early stages of disease. Most early cancer lesions appear as well-delimited, painless, erythroleukoplastic areas, whose principal signs of malignancy are the hardened texture, due to the partial loss of mucosal elasticity (Bagan & Scully 2011). Ulcerations occur over time, also some months after the early manifestations, often associated with irregular margins, and a progressive increase in extension and depth (**Figure 1.22**). With the advance of disease, persistent and progressive pain referred to adjacent tissues has been reported. The above-mentioned clinical features, with the maximum diameter of lesion less than 2 cm, are indicative of an early-stage OSCC. Of note, these initial lesions may also present exophytic, not defined tumor growth. The most significant relevant clinical features in this early stage of disease is the lack of defined margins and the tissue induration.

The lesions extension increases over time and can rapidly reach the maximum diameter of 4 cm, fixed as the limit for stage T2 tumors. At this stage, the patient manifests clear ulceration, with persistent pain, irradiated to adjacent regions.

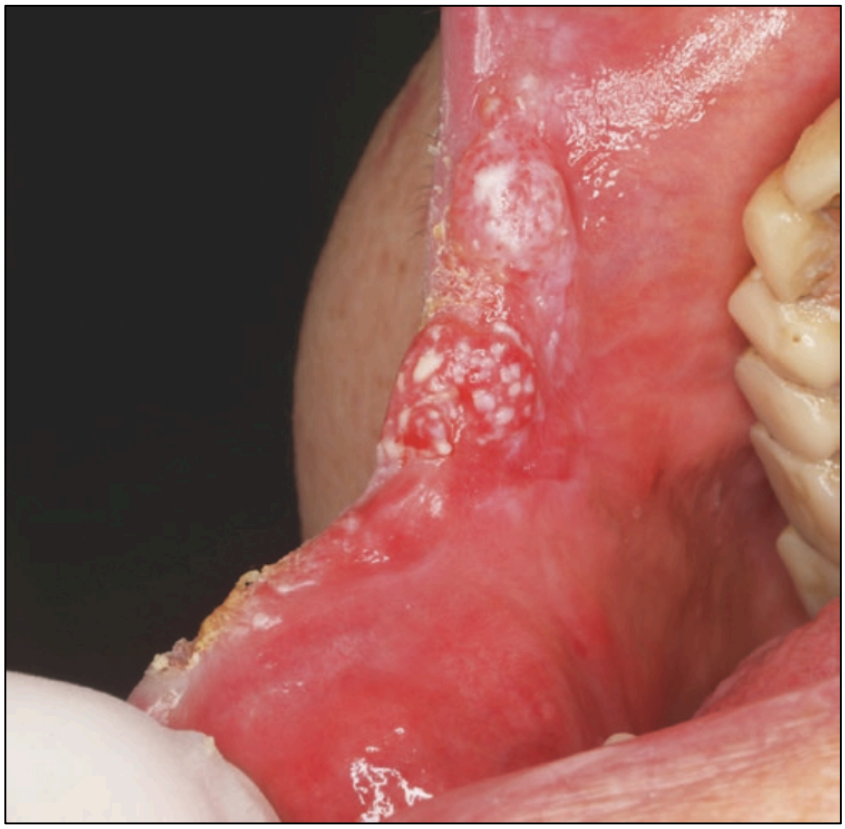

**Figure 1.22** Early-stage oral squamous cell carcinoma of the the retro-commissural area (*Source: Bagan JV, Bagan-Debon L. "Clinical Presentation and Differential Diagnosis of Oral Cancer" p.48, in Textbook of Oral Cancer, edited by Warnakulasuriya S, Greenspan JS, 2020. Springer International Publishing AG, Switzerland*).

### *1.4.2 Advanced stage*

Advanced-stage OSCC is classified by the extension of lesions over 4 cm or the infiltration of adjacent structures. At this point, patient exhibits an extensive ulcerated lesion, accompanied by in-depth and exophytic extension, with intensive pain (**Figure 1.23**). Narcotic agents are generally used at this stage to control the pain, often irradiated to near zone such as ear. Other additional symptoms and signs include bleeding, mobility of teeth, and paresthesias.

It is important to underline that head and neck cancers are characterized by a rapid neoplastic cell-mediated degradation of the basal membrane, and the consequent infiltration of the connective tissue. Thus, the neoplastic cells diffuse through the blood vessels, lymphatics, and nervous tissue. The metastases involving the neck regions are associated with a worse prognosis (Inglehart *et al.* 2014).

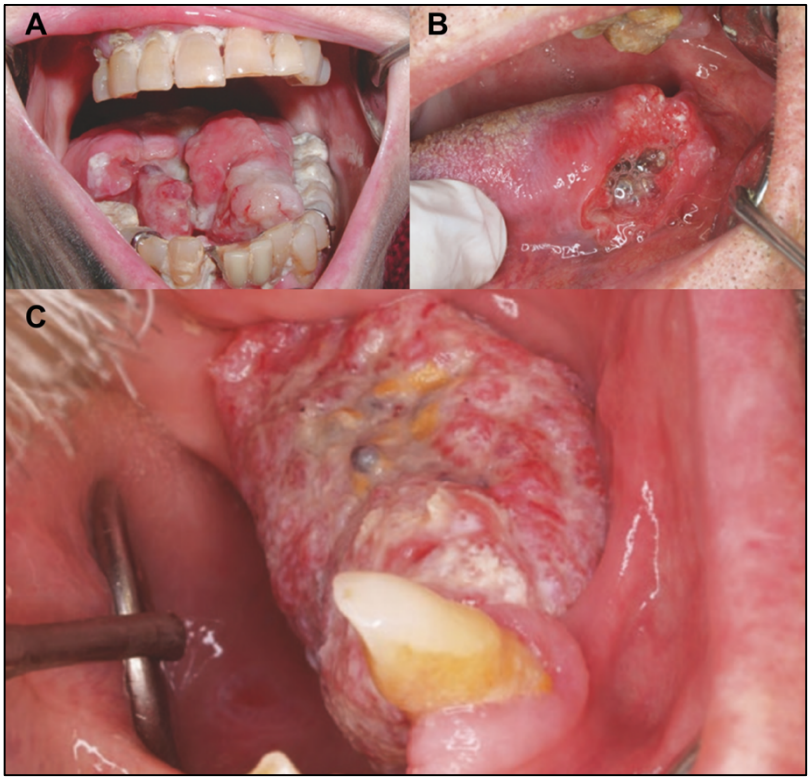


**Figure 1.23** Advanced-stage of oral squamous cell carcinoma of the floor of the mouth **(A)**, the lateral margin of the tongue **(B)**, and the lower gingiva **(C)**. (*Source: Bagan JV, Bagan-Debon L. "Clinical Presentation and Differential Diagnosis of Oral Cancer" p.51, 52, in Textbook of Oral Cancer, edited by Warnakulasuriya S, Greenspan JS, 2020. Springer International Publishing AG, Switzerland*).

## 1.5 TNM staging classification

Once a clinical and radiological diagnosis has been performed, the disease should be staged following the TNM classification, indicating the primary size and extent tumor (T stage) and the bone involvement and nodal metastasis (N stage) as well as any other distant metastasis (Edge *et al.* 2008, Martin & Webster 2012) (**Tables 1.1, 1.2**).

| Stage | Characteristics |
|---|---|
| **T Stage $T_x$** | Primary tumor cannot be accessed |
| **$T_0$** | No evidence of primary tumor |
| **T** | Carcinoma in-situ |
| **$T_1$** | GD<2 cm; DOI: <5 mm |
| **$T_2$** | 2cm<GD<4cm; 5mm<DOI<10 mm |
| **$T_3$** | GD: >4cm or any tumor with DOI >10 mm. |
| **$T_{4a}$** | Invasion of cortical bone or involvement of inferior alveolar nerve into deep (extrinsic) muscles of tongue or skin of face or maxilla |
| **$T_{4b}$** | Involvement of masticator space, pterygoid plates or skull base and or encasement of internal carotid artery |
| **N Stage $N_x$** | Regional lymph nodes cannot be accessed |
| **$N_0$** | No regional lymph node metastasis |
| **$N_1$** | Metastasis in a single ipsilateral lymph node; GD: <3cm |
| **$N_{2a}$** | Metastasis in a single ipsilateral lymph node: 3cm<GD<6cm |
| **$N_{2b}$** | Metastasis in multiple ipsilateral lymph nodes; GD<6cm |
| **$N_{2c}$** | Metastasis in bilateral or contralateral lymph nodes; GD<6cm |
| **$N_{3a}$** | Metastasis in a lymph node with GD>6cm |
| **$N_{3b}$** | Metastasis in any node with clinically overt ENE |
| **M stage $M_x$** | Distant metastasis cannot be accessed |
| **$M_0$** | No distant metastasis |
| **$M_1$** | Distant metastasis |

**Table 1.1** TNM classification of oral cancer. GD: great dimension; DOI: depth of invasion; ENE: extra-nodal extension (*Source: Bahadur S "Tumours of the oral cavity: diagnosis, assessment and staging Clinical Presentation and Differential Diagnosis of Oral Cancer" in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021, p.88. Springer International Publishing AG, Switzerland*).

| Stage | T stage | N stage | M stage |
|---|---|---|---|
| **I** | $T_1$ | $N_0$ | $M_0$ |
| **II** | $T_2$ | $N_0$ | $M_0$ |
| **III** | $T_3$ | $N_0$ | $M_0$ |
| | $T_1$ | $N_1$ | $M_0$ |
| | $T_2$ | $N_1$ | $M_0$ |
| | $T_3$ | $N_1$ | $M_0$ |
| **IV A** | $T_{4a}$ | $N_0$ | $M_0$ |
| | $T_{4a}$ | $N_1$ | $M_0$ |
| | $T_1$ | $N_2$ | $M_0$ |
| | $T_2$ | $N_2$ | $M_0$ |
| | $T_3$ | $N_2$ | $M_0$ |
| | $T_{4a}$ | $N_2$ | $M_0$ |
| **IV B** | $T_{4b}$ | Any N | $M_0$ |
| | Any T | $N_3$ | $M_0$ |
| **IV C** | Any T | Any N | $M_1$ |

**Table 1.2** Stage classification of oral tumors (*Source: Bahadur S "Tumours of the oral cavity: diagnosis, assessment and staging Clinical Presentation and Differential Diagnosis of Oral Cancer" in Management of Oral Cancers, edited by Bahadur S, Iyer S, 2021, p.89. Springer International Publishing AG, Switzerland*).

## 1.6 Treatment

Management of head and neck cancers presupposes a multidisciplinary approach including different medical specialists (Wheless *et al.* 2010). Indeed, overall survival (OS) results significant higher when patients are followed by specialized centers (Wuthrick et al. 2015). Early stage disease (stage I or II) requires surgical resection or radiation therapy to the primitive site. Loco-

regionally advanced disease (stage III or IV) is treated with a duplex approach including surgery and radiation therapy, associated or not with chemotherapy (Elkrief *et al.* 2020). Chemotherapy could be indicated due to the high risk of local recurrence and distant metastasis spread. Patients with evidence of metastasis need systemic therapy in addition to best supportive care. Prognosis is frequently poor with median survival varying from 6 to 12 months. At this stage of disease, therapeutic options integrate cytotoxic chemotherapeutic or molecularly targeted agents (Elkrief *et al.* 2020).

# 2. ORAL DYSBIOSIS

## 2.1 Introduction

The term “microbiota” refers to all the bacteria species organized within a specific environment while the term “microbiome” designs the collective genomes of microorganisms populating that environment (Turnbaugh *et al.* 2007, Dewhirst *et al*. 2010). Changes in microbiota homeostasis contributes to the progress of pathological conditions, including immune-mediated injures, metabolic disorders, chronic degenerative diseases, and cancer (Quigley 2017, Cornejo-Pareja *et al.* 2019, Baffy 2020, Fitzgibbon & Mills 2020). In regard to OSCC, alterations in oral microbiota stimulate cancer progression by modulating cell metabolism and influencing the synthesis of numerous cytokines (Meurman 2010, Vesty *et al.* 2018). Dysbiosis in resident microbiota promotes cancer pathogenesis by different mechanisms such as chronic inflammation and infections (Read & Douglas 2014, Wang *et al.* 2014), loss of epithelial barrier integrity (Pang *et al.* 2018), and collection of several epigenetic alterations (Allen *et al.* 2019) (**Figure 2.1**). The most common bacteria species associated with OSCC include *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Prevotella intermedia* (Mager *et al.* 2005, Katz *et al.* 2011, Atanasova & Ylmez 2014). In addition, A*ctinomyces*, *Clostridium*, *Enterobacteriaceae*, *Fusobacterium*, *Haemophilus*, *Porphyromonas*, *Prevotella*, *Streptococcus spp.* and *Veillonella* bacteria species have been linked with pre-cancerous lesions and oral cancer (Hu *et al.* 2016). Despite being proved to have some effects in carcinogenesis, oral microbiota exhibits a complex relationship with cancer that cannot be restricted to the assessment of a unique pathogen (Lee *et al.* 2017). Based on these assumptions, an increased number of studies looked for antitumor

effects of protective bacteria species provided as probiotics (Meurman 2010, Bann *et al.* 2017, Vivarelli *et al.* 2019). Advantages of probiotics derive from immune and inflammatory modulation, prevention of pathogen infections, reduced risk for cancer degeneration (Meurman 2010, Vivarelli *et al.* 2019).

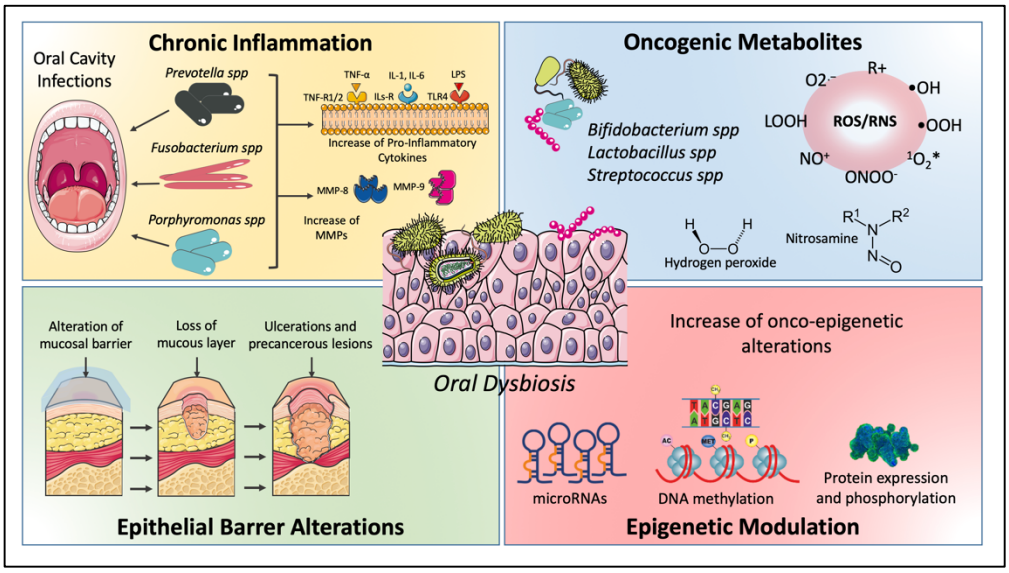


**Figure 2.1** Main mechanisms through which oral dysbiosis promotes carcinogenesis (*Source: La Rosa GRM, Gattuso G, Pedullà E, et al. Association of oral dysbiosis with oral cancer development. Oncol Lett. 2020;19: p. 3048*).

## 2.2 Changes in oral microenvironment promoted by dysbiosis

### *2.2.1 Chronic inflammation*

Inflammation is identified as a risk factor for almost 25% of human cancers (Multhoff *et al.* 2012). Periodontal disease (PD) is deemed to be one of the most common inflammatory conditions regarding the oral cavity and it has been indicated as potential contributing factor for cancer development (Corbella *et al.* 2018) (**Figure 2.2**). *Fusobacterium*, *Porphyromonas,* and *Prevotella* species are anaerobic oral bacteria involved in PD which maintain a status of chronic inflammation, through the synthesis of inflammatory mediators [i.e. interleukins (IL-1, IL-6, IL-17, IL-23), tumor-necrosis factor-α (TNF-α), and metalloproteinases (MMP-8, -9 and -13)] (Szkaradkiewicz & Karpiński 2013).

More in detail, some of these mediators (i.e. IL-6 and MMP-9) are associated with a worse prognosis and more aggressive tumor phenotype (Silva *et al.* 2017, Salemi *et al.* 2018).

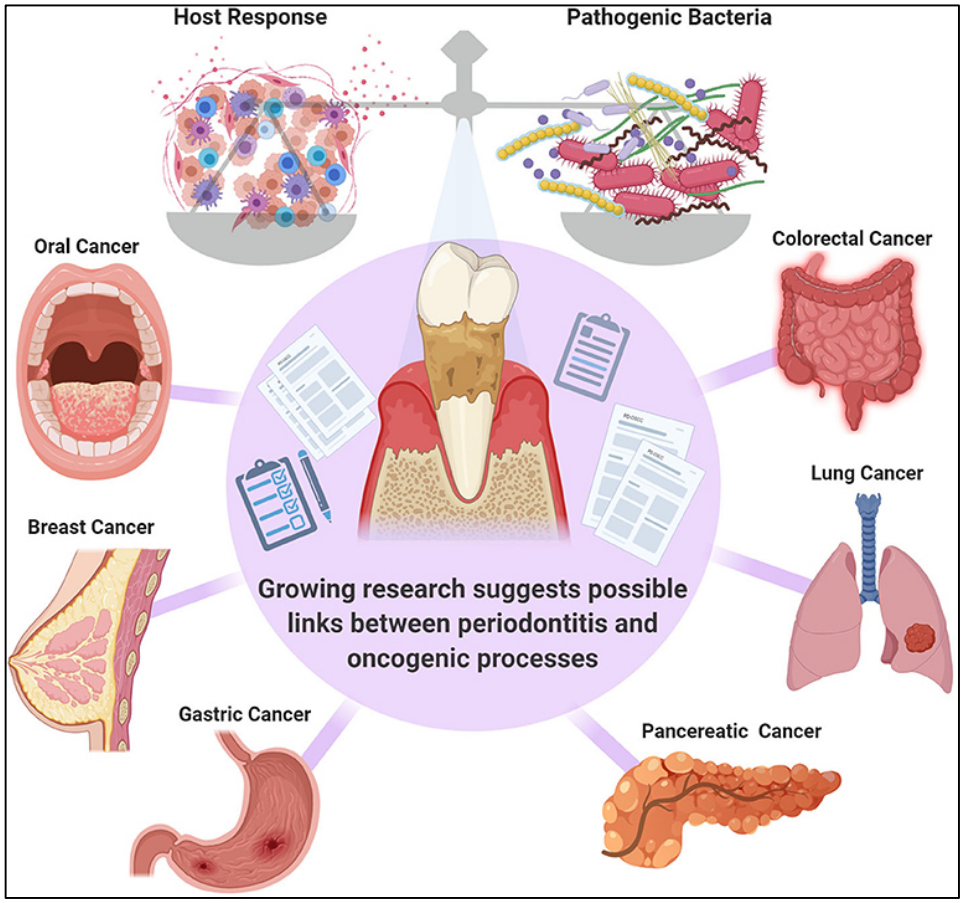


**Figure 2.2** Impaired microbiota-balance observed in periodontitis could favor carcinogenesis (*Source: Elebyary O, Barbour A, Fine N, et al. The Crossroads of Periodontitis and Oral Squamous Cell Carcinoma: Immune Implications and Tumor Promoting Capacities. Front. Oral. Health 2021;1: 584705, p.3).*

PD promotes a muthagen-enriched environment that can contribute to carcinogenesis (Galvão-Moreira & da Cruz 2016). In PD, the upregulation of *TP53* gene has destructive effects that undermine periodontal integrity. The *TP53* gene encodes the p53 tumor suppressor protein (known as the "guardian of the genome") whose alteration favors tumorigenesis (Memmert *et al.* 2016). *Porphyromonas gingivalis* can sustain tumor progression via STAT3 signaling that induces immunosuppressive myeloid-derived dendritic suppressor cells

(MDSCs) from monocytes, favoring oncogenic cell-proliferation and immune escape (Arjunan *et al.* 2018).

Interestingly, pathogenic bacteria affect immune cells behavior and other immune-regulatory networks (i.e. cytokines, chemokines, and growth factors) causing variations in immune surveillance (Hajishengallis & Lambris 2012).

#### *2.2.1.1 Immune cells response in inflamed periodontium*

In PD, many immune cells lose some of their characteristic defensive properties. These cells include, but are not limited to, neutrophils [known also as polymorphonuclear leukocytes (PMNs)] and cytotoxic T-lymphocytes which exhibit reduced defensins, defective neutrophil extracellular traps (NETs) formation and decreased IFN-γ production, respectively (Türkoglu *et al.* 2010, Uriarte *et al.* 2016, Petretto *et al.* 2019) (**Figure 2.3**).

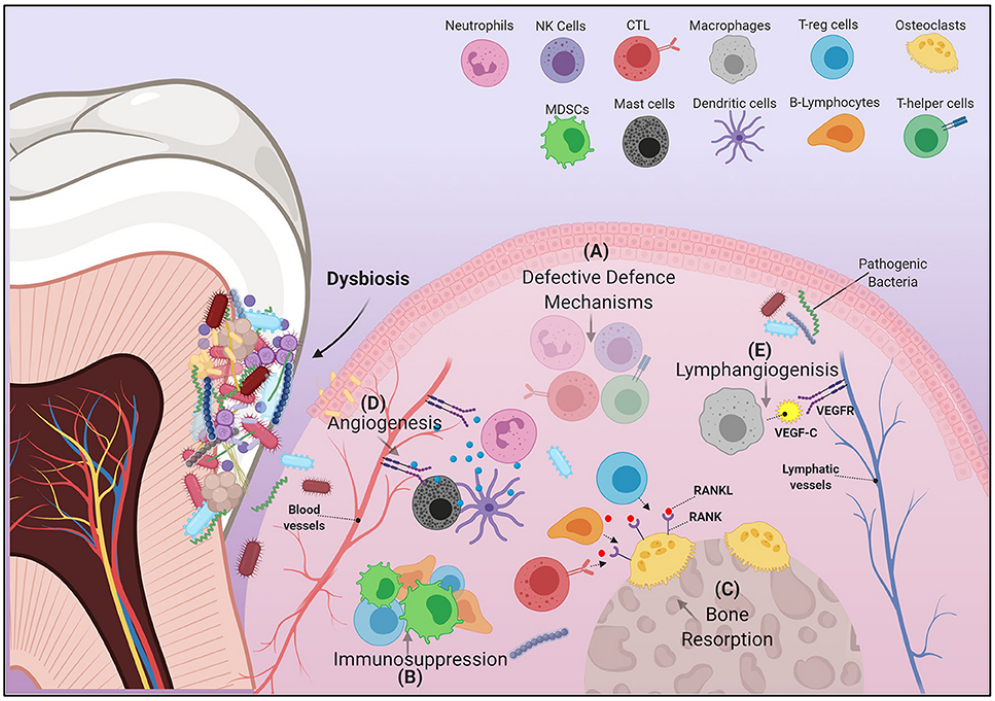


**Figure 2.3** Immune cells involved in the inflamed periodontium (*Source: Elebyary O, Barbour A, Fine N, et al. The Crossroads of Periodontitis and Oral Squamous Cell Carcinoma: Immune Implications and Tumor Promoting Capacities. Front. Oral. Health 2021;1:584705, p.5).*

All these PMN-secrets are related to the immune response and their dysregulation can support functional alterations, promoting cancer evolution (Elebyary *et al.* 2021).

In addition, impaired Th1 response was observed for T-helper cells and decreased granzyme and IFN-γ production for natural killer cells (Gaur *et al.* 2014, Dutta *et al.* 2015). The cytotoxic activity of these cellular lines results strongly jeopardized in PD due to immunosuppressive molecules in the periodontal inflammatory microenvironment. In this altered inflamed microenvironment, immune suppressive cells [i.e. myeloid-derived dendritic suppressor cells (MDSCs), T-regulatory cells (T-regs), B-regs (a subtype of B-lymphocytes)] are recruited to the inflammation site damaging immune surveillance (Lao *et al.* 2016, Alvarez *et al.* 2018, Dar *et al.* 2020).

Furthermore, periodontal inflammation and related bacterial pathogens are responsible for damage to the specialized tissues that take part of periodontium (i.e. epithelium, connective tissue, vasculature, and bone) (**Figure 2.4**). These changes can enhance the metastatic progression, survival, and proliferation of cancer cells, clarifying why the gingiva among the all oral soft tissues is the most recurrent site presenting metastatic oral carcinomas (Allon *et al.* 2014). Immune components involved such as T-regs, B-lymphocytes and cytotoxic T-lymphocytes determine an increased receptor activator of nuclear factor kappa-B ligand (RANKL) expression which determines the resorption of alveolar bone (Chen *et al.* 2014). Inflamed site is also characterized by an increased growth of periodontium vascular component mediated by several immune cells-factors. These factors include MMP-9, mostly produced by neutrophils and dendritic cells, and many angiogenic components released by mast cells [i.e. vascular endothelial growth factor (VEGF), tryptase, heparin, histamine, IL-8, basic fibroblast growth

factor] (Jotwani *et al.* 2010, Ng *et al.* 2011, Gaje *et al.* 2016, Tang *et al.* 2018, Wei *et al.* 2018). Finally, an expansion of lymphatic vessels is also observed, induced by VEGF-C.

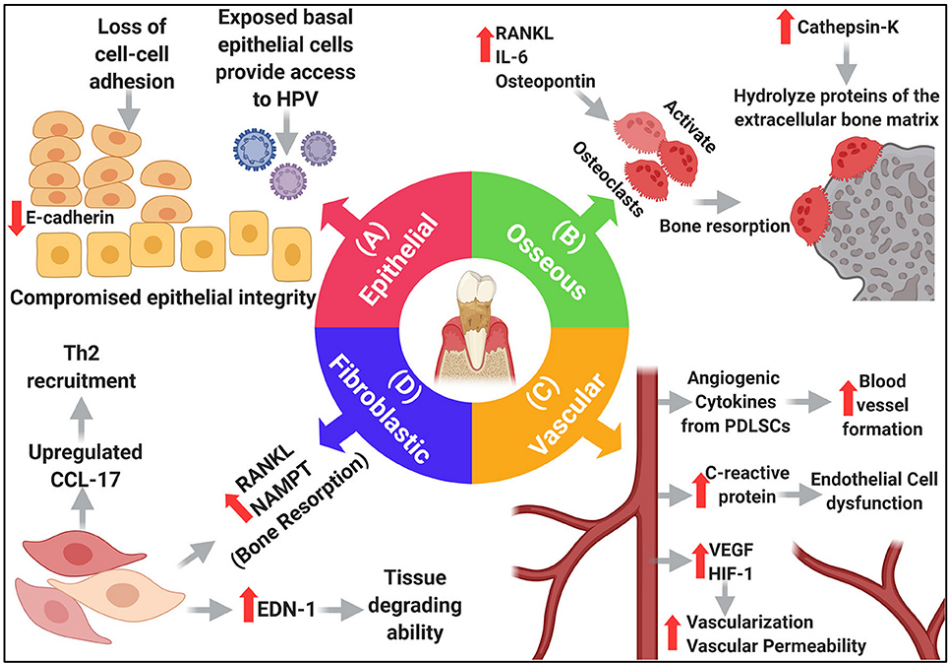


**Figure 2.4** Modifications in periodontal microenvironment due to chronic inflammation (*Source: Elebyary O, Barbour A, Fine N, et al. The Crossroads of Periodontitis and Oral Squamous Cell Carcinoma: Immune Implications and Tumor Promoting Capacities. Front. Oral. Health 2021;1:584705, p.11).*

### *2.2.1.2 Inflammation mediators*

The inflammatory factors including upregulated cytokines are able to affect different processes implicated in the modulation of cell metabolism and proliferation. For instance, Receptor for Advanced Glycation Endproducts (RAGE) protein expression is significantly modified by periodontal disease and can stimulate carcinogenesis (Katz *et al.* 2010). Furthermore, gram-negative bacteria are characterized by pro-inflammatory lipopolysaccharide endotoxin (LPS) which induces the synthesis of IL1-β, IL-6, and TNF-α by binding the Toll-Like Receptor (TLR) receptor of leucocytes (Zhang *et al.* 2000, Karpiński *et al.* 2019). These inflammatory cytokines favor the overexpression of other pro-inflammatory proteins promoting the release of phospholipase A2, prostaglandins

(PG), and acute phase proteins (Hou *et al.* 2003, Konopka *et al*. 2010). In addition, upregulation of IL-1 induces a pro-angiogenetic microenvironment, sustaining tumor spread (Voronov *et al.* 2003, Carmi *et al.* 2013). Tumor progression is also stimulated by the overexpression of IL-6 through upregulation of matrix-metalloproteinases, adhesion factors, and endothelial leukocyte adhesion molecules (Kossakowska *et al.* 1999).

De-regulated levels of TNF-α have been put in relation with modification of Wnt and factor nuclear factor κappa B (NF-κB) pathways, leading to tumor development (Rivas *et al.* 2008). Indeed, NF-κB functions as an immune-stimulant factor against tumor cells; nevertheless, abnormal activation of NF-κB (Vogelmann & Amieva 2007) promoted by oral dysbiosis and pathogens exposure, increases its protein expression acting as an oncogene in numerous cancers (Vogelmann & Amieva 2007, Karin 2009, Hoesel and Schmid 2013).

### *2.2.2 Synthesis of substances with oncogenic activity*

Several bacteria are able to produce substances including sulfur compounds, acids and free radicals, nitric and oxygen reactive species (ROS), as well as acetaldehyde exhibiting a carcinogenic action (Karpiński 2019).

Moreover, some bacteria can induce alterations of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase (NOS) activity leading to cumulative harmful substances such as reactive nitrogen species (RNS) with inflammatory properties (Hussain *et al.* 2003, Piao *et al.* 2016). Many peroxygenase oral microorganisms (i.e. *Bifidobacterium adolescentis*, *Lactobacillus acidophilus*, *L. fermentum*, *L. jensenii*, *L. minutus, Streptococcus gordonii*, *S. mitis*, *S. oligofermentans*, *S. oralis*, and *S. sanguinis*)

take part in this mechanism releasing hydrogen peroxide (H2O2) (Brauncajs *et al*. 2001, Abranches *et al*. 2018, Karpiński 2019).

Bacteria such as *Bacteroides* and *Firmicutes* species can ferment the host excessive protein into sulfides and nitrosamines that cause damage to oncogene or tumor suppressor genes (Carbonero *et al.* 2012, Bhatt *et al.* 2017). Moreover, other bacteria comprehending *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Peptostreptococcus stomatis* and *Streptococcus* lessen the environmental pH through the release of several types of acids (i.e. lactic, acetic, butyric, isobutyric, isovaleric, and isocaproic acids) (Karpiński & Szkaradkiewicz 2013, Senneby *et al*. 2017). These molecules sustain an acid microenvironment, suitable for tumor cell multiplication and progression (Lunt *et al.* 2009, Mazzio *et al.* 2010).

Interestingly, rise in the activity of superoxide dismutase (SOD) -enzyme which converts $O2^{-}$ in $H_2O_2$- was noted in tumor microenvironment (Yost *et al.* 2018). $H_2O_2$ can interact with $Fe2^{+}$ ions inducing the production of extremely reactive intermediates which cause DNA mutations and cancer cell proliferation by modulating cell cycle proteins expression (Franco *et al.* 2008).

A carcinogenetic mechanism mediated by microbiome tryptophanase activity has been also described. The enhancement of L-tryptophan degradation in secondary metabolites (i.e. indole, pyruvate, and ammonium) has been correlated with cancer development (Yost *et al.* 2018). These products are metabolized into agonist for the aryl hydrocarbon receptor (AHR) whose activation is involved in carcinogenesis (Murray *et al.* 2014). Similarly, other enzymes such as glutamate dehydrogenase (GDH) are high-expressed in oral cancer tissues and their alteration may provide significant variations in cellular redox state (Jin *et al.* 2015).

Finally, numerous oral microorganisms belonging to the *Streptococcus* species (*S. gordonii*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis*) (Pavlova *et al.* 2013), and *Candida* yeasts (Marttila *et al.* 2013) are responsible for metabolism of alcohol in acetaldehyde that possesses a carcinogenic potential activity (Vogelmann & Amieva 2007, Marttila *et al.* 2013).

### *2.2.3 Alteration of epithelial barrier integrity*

The interaction between dysbiosis and alteration of epithelial barriers supports carcinogenesis (Pang *et al.* 2018). Indeed, variations in anatomic configuration or in microbial constitution may induce an epithelial barrier dysfunction and microenvironment modifications (Schwabe & Jobin 2013). The resulting imbalance between epithelia/microbiota seems to favor infections, microbial diseases, and cancer progression (Seller & Morton 2014, Taur & Pamer 2016). Epithelial barrier alteration could be also sustained by pro-inflammatory factors associated with microbial dysbiosis. Besides the cancer progression, chronic inflammation promotes microbial translocation and modifies the microbiota selecting the growth of certain bacteria (Arthur *et al.* 2012, Elinav *et al.* 2013, Schwabe & Jobin 2013).

Microbial metabolites including ROS and hydroxyl radical and toxins such as cytolethal distending toxin (CDT) induce genomic damages through the neoplastic degeneration of epithelial cells. Furthermore, bacteria activate signal transduction pathways involved in epithelial cell proliferation by means of the virulence genes (i.e. AvrA virulence factor) and the transforming growth factor β (TGF-β). TGF-β is a pluripotent cytokine that controls epithelial barrier and immune response assuming a pivotal role in microbiota-epithelial interaction. TGF-β can be tumor-permissive stimulating the cell growth and blocking

apoptosis as well as it is responsible for immune-evasion of tumor antigens expressed by special microbes (Pang *et al.* 2018, White *et al.* 2010). Moreover, TGF-β acts as an immunomodulating factor preventing dendritic cells (DCs) and T-receptor, helping tumor cells to escape from immune surveillance (Yang 2010). Notably, the role of TGF-β signaling in tumorigenesis is also linked with the dysregulated inflammation microenvironment triggered by microbiota (Pang *et al.* 2018) (**Figure 2.5**).

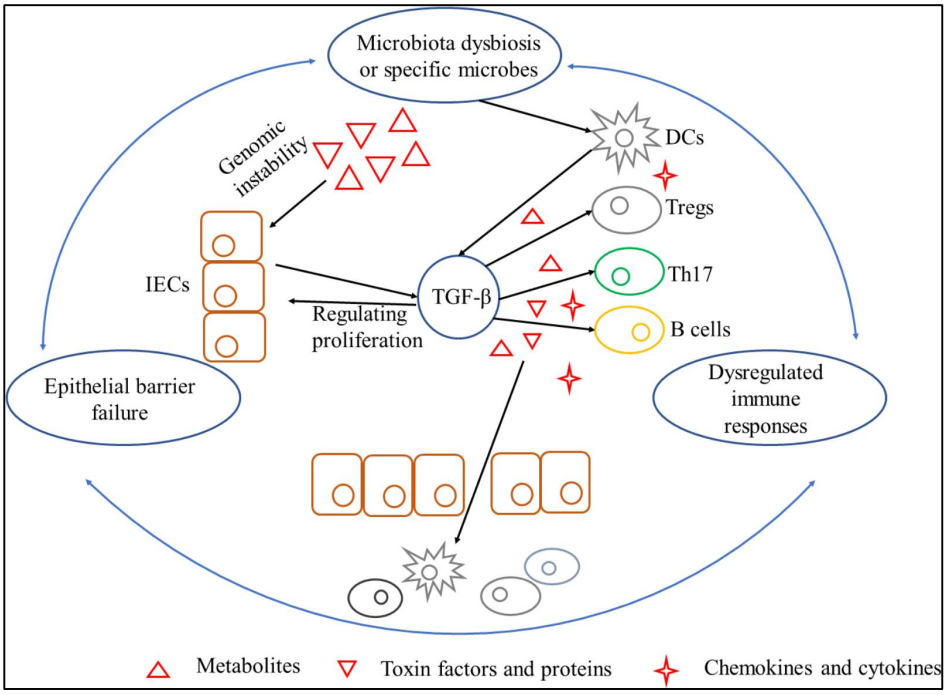


**Figure 2.5** Interactions between microbiota dysbiosis and TGF-β signaling in oncogenesis (*Source: Pang X, Tang YJ, Ren XH, et al. Microbiota, epithelium, inflammation, and TGF-β signaling: An intricate interaction in oncogenesis. Front Microbiol 2018;9:1353, p.6).*

### *2.2.4 Epigenetic modifications mediated by dysbiosis*

Environmental factors, including diet, life-style habits, and natural elements can modify the progression of several diseases including cancer through genetic and epigenetic variations (Pennisi *et al.* 2017, Lanza *et al.* 2018). Particularly, food consumption affects numerous cellular and molecular pathways acting in a multifactorial way (Soldati *et al.* 2018, Malfa *et al.* 2019). Conversely, several studies have found evidence that imbalance of nutrient consumption

and/or absorption is correlated with epigenetic changes able to promote specific pathologies, including cancer. The mechanism by which nutrients assumption could stimulate the onset of specific disease is well not established. Anyway, several studies pointed out that foods can influence the individual's redox state, the oral and gut microbiota, the DNA methylation status and the modification of microRNA (miRs) expression levels (Guillemin *et al.* 2017, Kadayifci *et al.* 2018, Murtaza *et al.* 2019). miRs are non-coding RNA constituted by 20-22 nucleotides, whose dysregulated expression is strictly associated with the progression of different tumors (McCubrey *et al.* 2017, Falzone *et al.* 2018, Candido *et al.* 2019). Role of non-coding RNA -mainly miRs- in carcinogenesis is addressed in the **Chapter 3**.

A number of studies have suggested a reciprocal interaction between microbiota and miRs (Dalmasso *et al.* 2011, Peck *et al.* 2017, Yuan & Subramanian 2019). Fecal miRs deriving from intestinal epithelial cells have demonstrated to penetrate some bacteria (i.e. *Fusobacterium nucleatum* and *Escherichia coli*) regulating the gene expressions and modifying the microbiota status (Liu *et al.* 2016). These preliminary results corroborate the potential role of miRs in regulation of gut microbiota and microbiome. Of note, several researches stated that fecal miRs can be originated from foods and absorbed by intestinal epithelia regulating the expression of host genes (Teodori *et al.* 2019, Zhang *et al.* 2019). These miRs are primarily planted exosome-derived even if there is growing evidence that also milk-derived miRs can be active (Wagner *et al.* 2015, Golan-Gerstl *et al.* 2017). It is reasonable to hypothesize that food-derived miRs are able to interact with oral and gut microbiota (Adami *et al.* 2018, Teng *et al.* 2018).

It is important to underline that also the resident microbiota may check the expression of specific miRs, thus confirming a reciprocal relationship between

miRs and microbiota and their ability to influence each other (Yuan *et al* 2019). Microbiota alters the miRs expression by producing different metabolites which can significantly impact on host-cell metabolism (Patrignani *et al.* 2014, Miro-Blanch & Yanes 2019). Future studies will have to clarify the mutual interaction between microbiota and miRs expression.

## 2.3 Bacteria with carcinogenic activity

Microbiota of oral cancer samples is more complex and diversified if compared with that of healthy individuals. Particularly, *Catonella*, *Dialister*, *Filifactor*, *Fusobacterium*, *Parvimonas*, *Peptococcus,* and *Peptostreptococcus* are the most high-expressed periodontitis-associated taxa bacteria in OSCC samples (Zhao *et al.* 2017) (**Figure 2.6**).

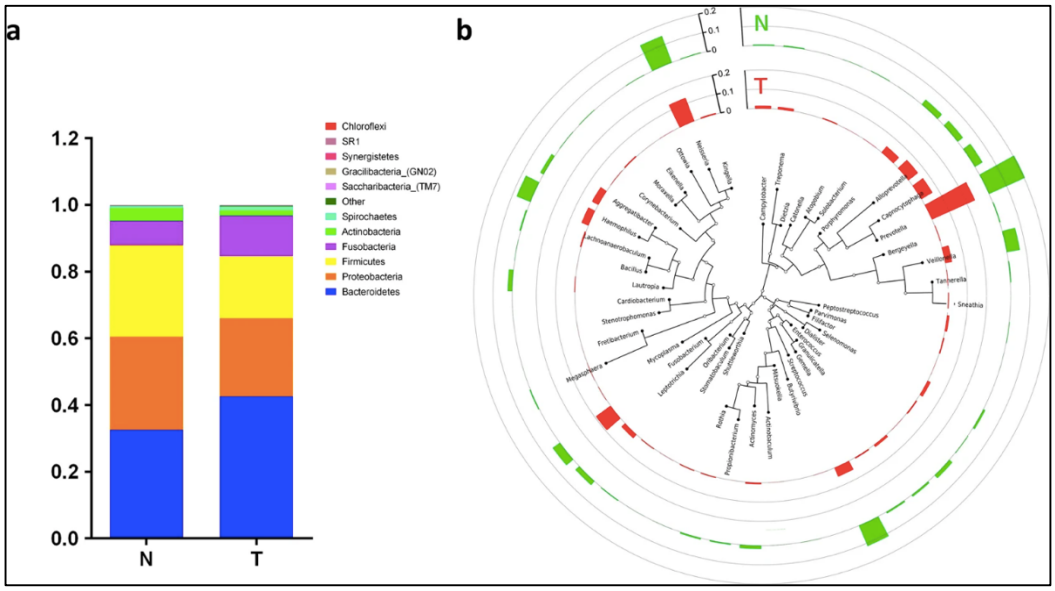


**Figure 2.6** Bacterial communities at the phylum and genus levels. (**a**) Composition of bacterial phyla in the normal and tumor groups; (**b**) representation of the 50 most present genera across all samples. N, normal samples; T, tumor samples (*Source: Zhao H, Chu M, Huang Z, Yang X, Ran S, Hu B, Zhang C and Liang J: Variations in oral microbiota associated with oral cancer. Sci Rep 2017;7:11773, p.3).*

Interestingly, numerous operational taxonomic units (OTUs) correlated with *Fusobacterium* –mainly *F. Nucleatum*- have been reported to be connected

with several cancers and suggested as a diagnostic indicator for OSCC (Yamamura *et al.* 2016, Zhao *et al.* 2017, Hsieh *et al.* 2018).

A meta-transcriptomic analysis of the oral microbiome in OSCC patients revealed a major expression of *Fusobacteria* transcripts in both tumor and peritumoral tissue samples in comparison to those of healthy participants. Moreover, *Fusobacteria* virulence factors have been correlated with the pathogenesis of oral cancer (Yost *et al.* 2018). These findings corroborate the previous results obtained by Nagy *et al.* (1998) who indicated some bacteria, including *Fusobacterium* sp., to be associated with keratinizing squamous cell carcinomas. Furthermore, microbiome alterations could be an indicator of cancer progression. The *Fusobacteria* quantity was significantly increased in oral cancer patients exhibiting a certain progression, with the major expression in stage 4 (7.92%) in respect of healthy controls (2.98%) (Yang *et al.* 2018a).

Lim *et al.* (2018) examined oral rinse to identify the microbiome alterations and their potential correlation with oral and head and neck cancers. A panel of bacteria (i.e. *Capnocytophaga*, *Corynebacterium*, *Haemophilus*, *Oribacterium*, *Paludibacter*, *Porphyromonas*, and *Rothia*) has been proposed as a tool to predict the risk of oral and oropharyngeal cancer, obtaining 100% and 90% sensitivity and specificity, respectively (Sasaki *et al*. 2005, Lim *et al.* 2018). These outcomes are in agreement with some previous findings (Mager *et al.* 2005, Sasaki *et al.* 2005, Katz *et al.* 2011, Pushalkar *et al.* 2012, Galvão-Moreira & da Cruz 2016, Lee *et al.* 2017) reporting *Capnocytophaga gingivalis*, *Peptostreptococcus* sp., *Porphyromonas gingivalis*, *Prevotella* sp., and *Streptococcus* sp. as the oral bacteria principally correlated with OSCCs. Conversely, the work of Yost et al. (2018) demonstrated that *Capnocytophaga gingivalis* was one of the most representative bacteria in healthy sites. The contrasting results are probably due

to the different samples evaluated, as well as to the number of cases enrolled (Yost *et al.* 2018).

Other oral bacteria associated with head and neck cancer development were *Streptococci*, especially *Streptococcus anginosus* (Shiga *et al.* 2001, Narikiyo *et al.* 2004, Mager *et al.* 2005).

Intriguingly, microbiota changes could be related to the genetic modifications in OSCC patients. An analysis of saliva samples showed an imbalance in the oral cavity taxa with *Bacteroidetes* and *Firmicutes* species highly expressed in three different groups of oral cancers clustered in accordance with the genetic mutational pattern. In addition, authors reported significant differences in microorganism's composition among the three groups, concluding that an association between OSCC mutations and microbiota changes could be plausible (Yang *et al.* 2018b).

Dissimilarity in bacterial constitution was also noticed between precancerous lesions and cancer samples (Lee *et al.* 2017). Remarkably, the genera *Bacillus*, *Enterococcus*, *Parvimonas*, *Peptostreptococcus*, and *Slackia* demonstrated a predictive value to distinguish between precancerous and cancerous lesions (Lee *et al.* 2017).

## 2.4 Probiotics as anti-tumoral agents

Probiotics are live microorganisms that are considered for health profits when consumed or applied to the body. Probiotics may be constituted by a variety of microorganisms, with *Lactobacillus* and *Bifidobacterium* groups the most representative (Goodman & Gardner 2018, Tsai *et al.* 2019). Anti-tumoral properties of probiotics have been widely investigated. They contrast the mutagenic effects of detrimental substances influencing the expression of proteins

implicated in cell proliferation, apoptosis, inflammation, or immune system activation (Kumar *et al.* 2010). Numerous *in vitro* studies conducted on tumor models demonstrated the anti-proliferative and pro-apoptotic properties of probiotics including *Bifidobacterium longum*, *L. acidophilus* and *L. casei* species (Lee *et al.* 2004, Yu *et al.* 2016b). *Lactobacillus acidophilus 606* (Kim *et al.* 2010) and *Lactobacillus rhamnosus GG* (Vivarelli *et al.* 2019) are other probiotics exhibiting anti-neoplastic activities in colorectal cancer cell lines.

Anti-tumoral effects of probiotics on oral cancer are not completely established. A recent study investigated the effects of *Lactobacillus rhamnosus GG* (LGG) in OSCC cell lines to potentiate the antineoplastic effects of geniposide, a derivate of *Gardenia jasminoide* with anti-tumoral properties (Wan *et al.* 2014, Cheng *et al.* 2017). In agreement with the results obtained, the combined treatment reduced the apoptotic rate of cancer cells suggesting its potential use in clinical practice. Asoudeh-Fard *et al.* (2017) proposed also *Lactobacillus plantarum* as antineoplastic agent due to its ability to hamper and activate the mitogen-activated protein kinases (MAPKs) and phosphatase and tensin homolog (PTEN) pathways, respectively. Indeed, PTEN and MAPKs are associated with the suppression and the cancer initiation, respectively.

Furthermore, another study stated that *Acetobacter syzygii* strain secretions exhibit anticancer activity stimulating the apoptosis in oral cancer cells. Of note, *Acetobacter syzygii* products were not implicated in the dysbiosis of the epithelial cell lines (Aghazadeh *et al.* 2017).

These findings corroborate the potential opportunity represented by the probiotics in controlling cancer development also in the oral tissues and support further studies on the effects of probiotics in OSCC progression.

# 3. MOLECULAR PATHWAYS INVOLVED IN OSCC DEVELOPMENT

## 3.1 Introduction

The impaired expression of numerous genes and the consequent loss of coordination of their downstream signaling pathways has been connected with oral cancer development and progression. The accumulation of multiple genetic and epigenetic alterations promotes the carcinogenic transformation of a single cell or a clone of cells into a malignant tumor. These key genes include some normal genes (known as proto-oncogenes) that when modified act as oncogenes, leading to persistent and uncontrollable cell proliferation. Conversely, tumor suppressor genes (*TSGs*) that normally avoid the growth of deregulated cells when are altered allow cell multiplication and avoidance of apoptosis (Hahn *et al.* 2002, Khan *et al.* 2013) (**Figure 3.1**). Most oral cancers are squamous cell carcinomas deriving from lining epithelia, which means that their behavior is similar to that of other human squamous cancers. The principal genetic alteration in this subtype of cancer regards the oncogenes and the cellular pathways involved (i.e. cell proliferation, differentiation, apoptosis, telomere maintenance, invasion, and angiogenesis). Nevertheless, model of carcinogenesis is not unique for all oral cancers and the identification of the molecular pathways involved could have significant and beneficial implications for diagnosis, prognosis, and treatment planning of oral cancers.

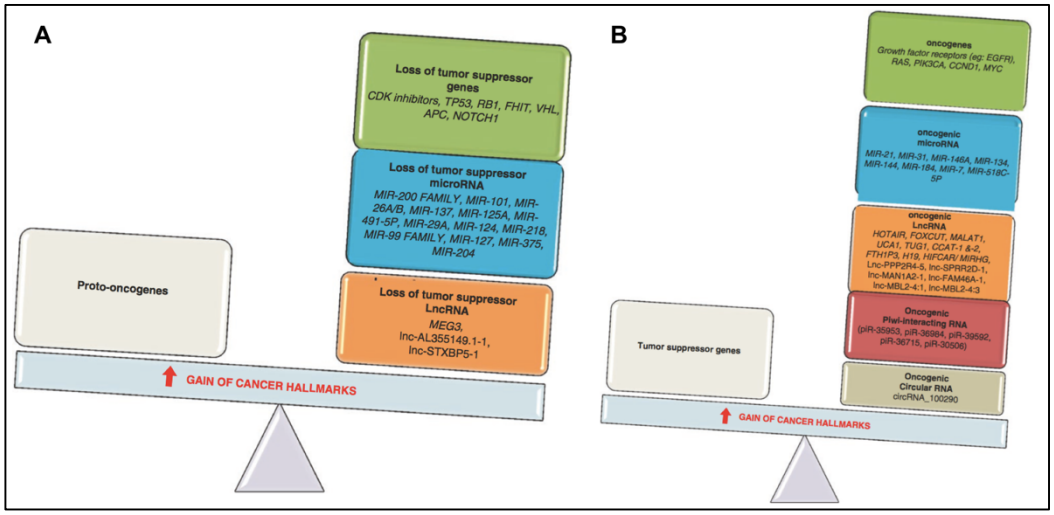


**Figure 3.1** Unbalance between tumor suppressor and oncogenic molecules promotes cancer initiation and progression. The loss of this balance could be due to either loss of function in tumor suppressors **(A)** and/or gain of function in oncogenes **(B)** (*Source: Panta P, Manavathi B, Nagini S. "Genetics and Molecular Mechanisms in Oral Cancer Progression" pp. 41, 42 in Oral Cancer Detection edited by Panta P, Andreadis D, 2019. Springer International Publishing AG, Switzerland)*.

## 3.2 Genetic profile of oral cancer

### *3.2.1 Mechanisms of genomic instability*

Two principal pathways for oral cancer development and/ or progression induce genomic instability. First, the tumor suppressor pathway for aneuploid cancer is marked by chromosomal instability that activates oncogenes and inactivates *TSGs*. Second, the mutator pathway for (pseudo)diploid cancer is marked by instability of microsatellites, which are simple, and repetitive sequences in DNA. Interaction between the two pathways is identified to be critical for the development of OSCC (Panta *et al.* 2019). "Microsatellites" are DNA stretches of 1–5 nucleotides repeated 5–100 times ubiquitously in the genome. The mismatch repair (MMR) system prevents slippage by DNA polymerase on microsatellites during replication. Defects in the MMR regions have been observed in oral cancer causing a microsatellite instability which may be considered crucial for cancer progression.

Chromosomal instability includes chromosomal alterations such as "loss of heterozygosity" (LOH) or "aneuploidy" (i.e. abnormal chromosomal number). LOH induces the loss of an entire gene and surrounding chromosomal elements. It is very common that chromosomal aberration to be found in oral cancer and usually regard a TSG, determining the loss of cell proliferation' control. Loss of chromosome 3p is an early event in the progression of dysplasia and malignancy and involves tumor suppressor genes including von Hippel-Lindau (*VHL*), Fragile Histidine Triad (*FHIT*), and Ras Association Domain Family Member 1 (*RASSF1*). Reduced expression of *FHIT* is a negative prognostic factor for survival rate (Kakkar *et al.* 2020). Another locus frequently lost (about 50% of cases) is chromosome 17p, where another tumor suppressor gene -tumor protein 53 (*TP53*) known also as "the guardian of the genome"- is represented. *TP53* codes for a transcription factor "tumor protein p53" which is responsible for transcription-mediated activation of apoptosis, G1-S cell cycle arrest, inhibition of angiogenesis, DNA repair, and genetic stability (**Figure 3.2**).

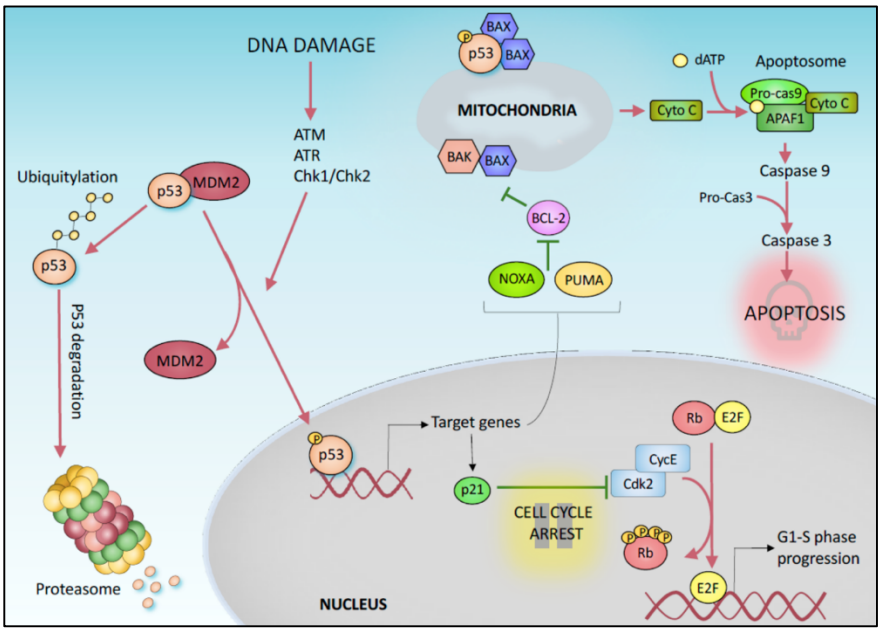


**Figure 3.2** p53-correlated DNA damage response (*Source: Pitolli C, Wang Y, Candi E, et al. p53-Mediated Tumor Suppression: DNA-Damage Response and Alternative Mechanisms. Cancers (Basel). 2019;11:1983, p.2*).

This alteration is frequent in invasive carcinoma suggesting that it occurs late in the disease progression (Perez-Ordoñez *et al.* 2006).

Another genetic alteration occurring during oral cancer development refers to "single nucleotide polymorphisms" (SNPs) which are genetic modifications that increase the susceptibility to cancer. SNPs target many genes implicated in carcinogen detoxification and folate metabolism, DNA repair [ataxia telangiectasia mutated (ATM)], inflammation, cell cycle regulation and proliferation, immune activity, and invasion (Bau *et al.* 2010, Multani *et al.* 2016, Shridhar *et al.* 2016b).

Interestingly, the genetic signature for oral cancer susceptibility has been demonstrated in a recent study. Genome-wide association studies (GWAS), and next-generation sequencing (NGS)-based approaches permitted to identify 28 chromosomal loci amongst an amount of 264, associated with oral cancer (Sharma *et al.* 2017).

### *3.2.2 Proto oncogenes*

Proto-oncogenes are genes that encode proteins responsible for cell growth and differentiation. Proto-oncogenes activation elicited by point mutations, chromosomal translocations, DNA rearrangements, and gene amplification causes neoplastic transformation. OSCC development has been linked with gain of function or increase in copy number of oncogenes sited at 1q, 3q, 5p, 7q, 8q, 9q, 11q, 12p, 14q, and 15q (Panta *et al.* 2019).

Among oncogenes, Epidermal Growth Factor Receptor (*EGFR*) and Cyclin D1 (*CCND1*) are the most recurrent mutated genes in the advancement of SCC. *EGFR* gene, situated on chromosome 7p, is frequently overexpressed in the head and neck SCC, coding for excessive cell growth. Epidermal growth factor

(*EGF*), a known ligand for this receptor, triggers *EGFR* causing the phosphorylation of several key substrates which participate to Phosphatidylinositol 3-Kinase/Serine/Threonine Protein Kinase/ Protein Kinase B (*BPI3K/Akt*), *MAPK*, Janus Kinase/Signal Transducers and Activators of Transcription (*JAK/STAT*), and Kirsten Rat Sarcoma/V-Raf Murine Sarcoma Viral Oncogene Homolog B/Mitogen Activated Protein-Extracellular Signal Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase (*KRAS/BRAF/MEK/ERK*) signaling pathways responsible for cancer hall-marks acquisition (Seshacharyulu *et al.* 2012, Lin *et al.* 2015, Concha-Benavente *et al.* 2016, Huang *et al.* 2016). In addition, the mutant receptor is active also in the lack of *EGF* producing an aberrant activation of Phosphoinositide 3-Kinase (*PI3K*)/*Akt*/Mammalian target of Rapamycin (*mTOR*) pathway that causes the transcription of *NF-κB* responsible for several downstream affects (Panta *et al.* 2019). The *PI3K/AKT/mTOR* is a pivotal cellular pathway that regulates cell cycle, cell growth, and proliferation (**Figure 3.3**).

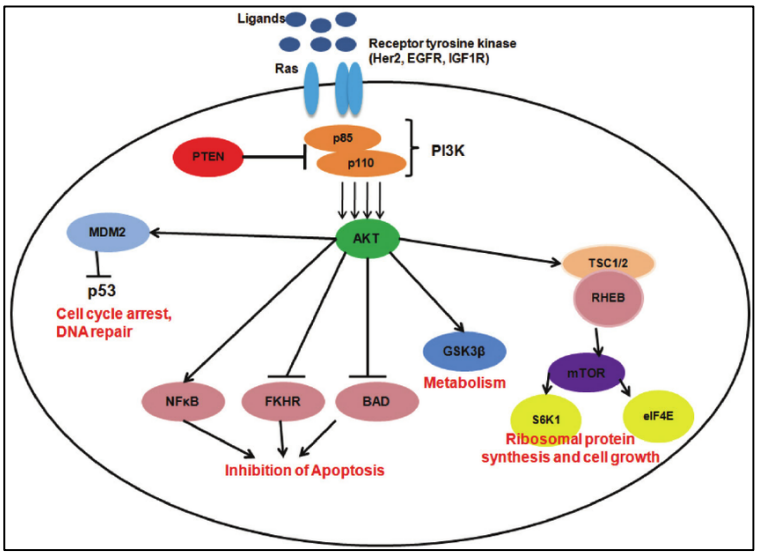


**Figure 3.3** PI3K/Akt/mTOR pathway. (*Source: Gaikwad SM, Ray P. Non-invasive imaging of PI3K/Akt/mTOR signalling in cancer. Am J Nucl Med Mol Imaging. 2012;2:p.420*).

Dysregulation of this pathway is demonstrated in more than 60% of head and neck SCC. Alterations in the Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*), *PTEN*, and Phosphoinositide-3-Kinase Regulatory Subunit 1 (*PIK3R1*) genes in this pathway promote cancer progression (Vander Broek *et al.* 2015). Furthermore, the downstream ligand of *EGFR* - transforming growth factor alpha (*TGF-alpha*)- is also commonly overexpressed in head-neck SCC, causing as a clinical result worse outcomes and resistance to chemotherapy (Dotto & Rustgi 2016). Thus, "*EGFR* activation is a primary cell surface signal in several cancers" (Panta *et al.* 2019).

Other receptors significantly upregulated in OSCC comprehend Insulin-Like Growth Factor 1 Receptor (*IGF1R*), Glucose Transporter 1 (*GLUT- 1*), Nerve Growth Factor Receptor (*NGFR*), and Fibroblast Growth Factor Receptor (*FGFR, FGFR-1, FGFR-2*) (Stadler *et al.* 2008, Xie *et al.* 2016).

Approximately half of SCC cases exhibited an amplification of the 11q13 locus with the subsequent overexpression of *CCND1*. In physiological conditions, *CCND1* checks the cell cycle arresting the G1–S transition through the phosphorylation and deactivation of the *Rb* gene. Thus, *CCND1* overexpression promotes cell cycle progression by preventing the checkpoint from the G1 to S phase. Amplification of 11q and the *CCND1* overexpression have been connected to worse outcomes such as lymph node metastases (Miyamoto *et al.* 2002).

### *3.2.3 Tumor suppressor genes*

Loss of function mutations in *TSGs* is very frequent in oral cancer, and generally refers to *TSGs* sited at 3p14, 4q, 5q, 6p, 6q, 7q, 8p, 9p, 9q, 10q, 11q, 13q14.2, 14q, 17q, 18q, 20q, 21q, and 22q. Some chromosomal sites -known as fragile sites- are highly susceptible to mutations which appear to be favored by

tobacco chewing and smoking (Panta *et al.* 2019). Inactivation of *TSGs* is caused by different mechanisms including promoter methylation, point mutations, chromosomal rearrangements, or loss of heterozygosity (Ha & Califano 2006, Stadler *et al.* 2008). The main *TSGs* involved in OSCC include *TP53*, retinoblastoma (*RB*), cyclin-dependent kinase (*CDK*) inhibitors, *FHIT*, adenomatous polyposis coli (*APC*), von Hippel-Lindau (*VHL*) syndrome, Notch (Drosophila) homolog 1 (*NOTCH-1*) (Zhanga *et al.* 2018, Panta *et al.* 2019). Deleted in oral cancer-1 (*doc-1*) has been indicated as a specifically tumor suppressor gene in oral carcinogenesis (Todd *et al.* 1995).

## 3.3 Epigenetic alterations

The term "epigenetics" refers to heritable modifications in gene expression that do not induce variations in the DNA sequence (Lin *et al.* 2017). Epigenetic modifications -DNA methylation, acetylation, histone modifications, chromatin remodeling, and noncoding RNA (ncRNA) mechanisms for gene silencing- assume a pivotal role in the development and progression of OSCC (Al-Kaabi *et al.* 2014).

DNA methylation is the most frequent mechanism, where the DNA strand is spotted with tiny molecules (i.e. methyl groups) changing the genes structure and their interaction with cellular environment. DNA hypermethylation can prevent binding of transcription factors to DNA resulting in the inactivation of *TSGs* responsible for the regulation of cell proliferation. This mechanism has been extensively described in head and neck squamous cell carcinomas, involving cyclin dependent kinase inhibitor 2A (*CDKN2A*) and O6methylguanineDNA methyl-transferase (*MGMT*) genes among others (Kato *et al.* 2006). Genes implicated in cell cycle regulation (p16, p15, and p14), cell-cell adhesion (E-

cadherin), Wnt signaling pathway (*APC*, *WNT* inhibitory factor 1, *RUNX3*), DNA repair (*MGMT*, MutL homolog 1), and apoptosis [apoptosis-associated death-associated protein kinase (*DAPK*)], as well as *p73*, *PTEN*, and Ras association family (*RASSF*) 1A, are the most frequently hypermethylated genes in OSCC (Demokan & Dalay 2011).

Alternatively, hypomethylation can activate proto-oncogenes causing genomic instability. Acetylation, deacetylation, and methylation are translational histone modifications which can cause conformational changes in DNA structure. Thus, histone modifications can either enhance or hinder binding of transcription factors to the promoter. Overexpression of histone deacetylase-1, one of the principal epigenetic reprogramming protein involved in silencing various growth regulatory pathways and proapoptotic programs, has been demonstrated in OSCC (Panta *et al.* 2019). The ncRNA controls gene expression at the post-transcriptional level by degrading or repressing the mRNA transcript to block translation (Gasche & Goel 2012).

### *3.3.1 Noncoding RNA (ncRNA)*

Less than 3% of the genome encodes proteins and approximately 75% is transcribed in RNAs that exhibit no coding potential (Panta *et al.* 2019). Consequently, research interest has recently concentrated on the role of these ncRNAs. They are classified basing on size and the arbitrary 200 nucleotides cutoff, in: small ncRNAs, which include miRs and Piwi-interacting RNAs (piRNAs); the longer ncRNAs that refer to long noncoding RNAs (lncRNAs), and circular RNAs (circRNA) (Soga *et al.* 2013, Gomes *et al.* 2017).

### *3.3.2 microRNAs*

MicroRNAs are small ncRNAs, made from 18 to 25 nucleotides, principally found in the intergenic and intronic regions (Soga *et al.* 2013, Gomes *et al.* 2017) (**Figure 3.4**).

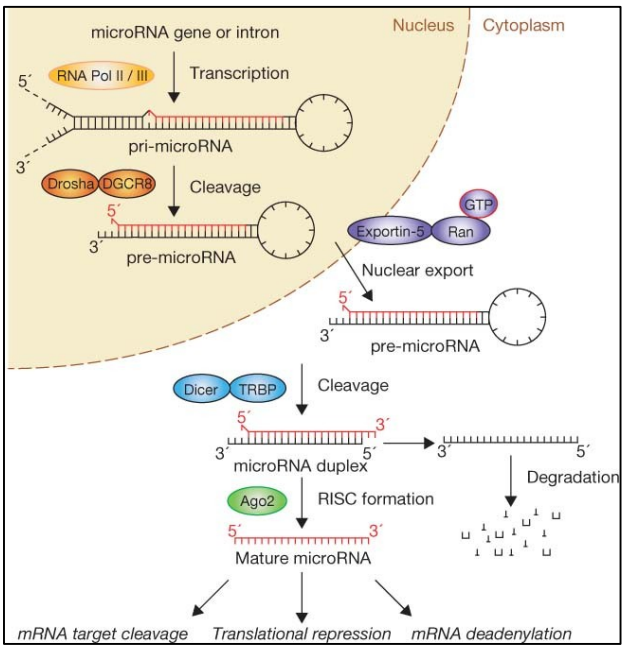


**Figure 3.4** Pathway of miRs processing (*Source: Winter J, Jung S, Keller S, et al. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009;11:229).*

Each miR can modify gene expression and influence, consequently, key biological processes such as proliferation, apoptosis, and cell differentiation (Min *et al.* 2015). Because of their implication in numerous biological processes, their dysregulation is involved in oncogenic transformation, including oral cancer initiation and progression (Philipone *et al.* 2016). Some miRs regulate *P13K/Akt/NF-κB* signaling, others are able to hinder telomerase or induce epithelial–mesenchymal transition (EMT) and angiogenesis. Furthermore, miRs regulate important signaling pathways implicated in carcinogenesis by targeting oncogenes and *TSGs*. For instance, 14 *TSGs* are targets of miR-21, and 21 oncogenes are targets of miR-16 (Panta *et al.* 2019). Many oncogenes -*CCND1*, *MYC*, Harvey Rat Sarcoma Viral Oncogene Homolog (*HRAS*), *KRAS*, *CDK-4*,

*CDK-6*, high-mobility group AT-hook 2 (*HMGA2*)- and *TSGs* including *TP53, PTEN* are subjected to the control of multiple miRs (Philipone *et al.* 2016). Altered expression of miRs is thus correlated with clinic-pathological variables and has diagnostic and prognostic value in OSCC (Manasa *et al.* 2017).

#### *3.3.2.1 Oncogenic miRs*

Numerous miRs including miR-21, miR-31, miR-146a, miR-134, miR-184, miR-7, miR-127, and miR-518c-5p are significantly upregulated in OSCC (Hung *et al.* 2014). Many of these miRs, such as miR-21, miR-146, miR-184, and miR-7 regulate expression of genes involved in cell migration, apoptosis, and proliferation (De Sarkar *et al.* 2014, Hedbäck *et al.* 2014, Min *et al.* 2015). Hypoxic environment is able to induce the tumor cells to synthetize miR-21-rich exosomes which stimulate pro-metastatic effects in the normoxic cells, and promote progression of OSCC through exosome interactions (Li *et al.* 2016).

Moreover, in animal models and cell lines, miR-518c-5p has been reported to be able to regulate growth and metastasis (Kinouchi *et al.* 2014). Several mechanisms mediate functional effects of miRs and result altered in carcinogenesis.

The expression of miR-31 is correlated with *VEGF* expression (Hung *et al.* 2014, Lu *et al.* 2016) and the functional effects of miR-31 are regulated by fibroblast growth factor (*FGF-3*) and Rho-A leading to proliferation and migration (Chang *et al.* 2013). miR-146a is upregulated by the *NF-κB* pathway and "miR-146a-NF-κB" loop is suggested to connect inflammation with oral cancer (Min *et al.* 2015). With regard to miR-127, it is connected to cell proliferation and senescence through upregulation of the oncogene B-Cell Lymphoma 6 (*BCL-6*) (Lajer *et al.* 2011). mi-134 acts by affecting tumor

suppressor protein WW domain-containing oxidoreductase (*WWOX*) that interacts with other binding partners to regulate several biological processes (Pimenta *et al.* 2006). As concerns miR-144, functional pathway analysis recognized the tumor suppressor *PTEN* as a target of miR-144 (Manikandan *et al.* 2016). Finally, cell-based assays showed that miR-518c-5p is a downstream target of the stromal cell-derived factor (SDF)-1/chemokine receptor CXCR4 axis that is involved in metastasis development (Gelmini *et al.* 2008, Kinouchi *et al.* 2014).

Interestingly, upregulated miRs could be predictors of OSCC outcomes. For example, the overexpression of miR-21 combined with a late stage tumor, and keratinization state have been suggested as indicators of poor prognosis in OSCC (Jung *et al.* 2012). In addition, miR-31 has been proposed a potential marker for detection of high-risk OPMD (Hung *et al.* 2016) and a high expression of miR-134 as an independent predictor of poor survival in OSCC (Pimenta *et al.* 2006). miR-184 was among a group of miRs whose reduced expression could be a predictor for assessment of treatment outcome (Santhi *et al.* 2013). Nevertheless, Manikandan *et al.* (2015) analyzing 42 OSCC specimens, reported that miR-184 was downregulated in tumor specimens. These differences could be due to the different methodological conditions as well as to the different number of samples analyzed.

#### *3.3.2.2 Tumor suppressor miRs*

Some miRs including miR-200 family, miR-101, miR-26a/b, miR-137, miR-125a, miR-491-5p, miR-29a, miR-124, miR-218, miR-99 family, miR-375, miR-204 can be tumor suppressors (Min *et al.* 2015). These miRs are generally downregulated in tumor tissues through hypermethylation.

Down-regulation or loss of these miRs negatively affects many cellular pathways involved in cancer progression. miR-200 family modulates E-cadherin repressors: zinc finger E-box binding homeobox 1 (*ZEB-1*) and zinc finger E-box binding homeo- box 2 (*ZEB-2*) (Lei *et al.* 2015). Thus, downregulation of miR-200 family is crucial for oral cancer progression. In addition, miR-101 is inversely correlated with *ZEB-1* and its under-expression stimulates cells proliferation and cellular invasion (Baolei *et al.* 2016). Cancer proliferation and progression is also regulated by loss of miR-26a/b through: transmembrane protein 184B (*TMEM184B*), E-cadherin, and EGF LAG seven-pass G-type receptor 1 (*CELSR1*) genes (Fukumoto *et al.* 2015). Furthermore, the tumor suppressor function of miR-137 occurs by targeting specificity protein (*SP1*) zinc finger transcription factor (Sun *et al.* 2016). The methylation of miR-137 has been documented in many studies on OSCC and OPMDs (Langevin *et al.* 2011, Dang *et al.* 2013) and can serve as a relevant prognostic marker (Langevin *et al.* 2011).

miR-125a is involved in the progression of OSCC by targeting the estrogen-related receptor alpha (*ESRRA*) that belongs to the nuclear receptor superfamily responsible for cancer cell migration and invasion. *ESRRA* results to be upregulated in tissues with reduced miR-125a and large energy requirement (Tiwari *et al.* 2014). In regard to miR-491-5p, its under-expression affects OSCC progression through its target G-protein-coupled receptor kinase-interacting protein 1 (*GIT1*), a scaffold protein associated with paxillin. Paxillin is an adaptor protein which supervises cell motility by assembling cytoskeleton and it is able to modify the levels of miR-218. The *GIT1*/paxillin complex controls the cell adhesion and migration (Huang *et al.* 2014).

Tumor invasion and metastasis have demonstrated to be affected by reduced levels of miR-124 and miR-99 family downregulation (Hunt *et al.* 2011,

Chen *et al.* 2012). Low mir-124 promotes tumor cell proliferation upregulating the integrin beta-1 (*ITGB1*); mir-99 family targets numerous genes including homeo-box A1 (*HOXA1*) that regulates proliferation, cell migration and apoptosis (Chen *et al.* 2013). miR-375 is strongly down-regulated in OSCC and OLP, strengthening the potential association between chronic inflammation conditions such OLP and carcinogenesis (Shi *et al.* 2015). Another miR commonly down-regulated in numerous cancer models including oral is miR-204 which has been proved to promote oral cancer stemness and lymph node infiltration in animal models (Yu *et al.* 2016c).

In addition to the above mentioned miRs, both upregulated and down-regulated, many others miRs are being studied to establish their role as oncogenes or tumor suppressors in oral cancer development. Consequently, miRs are of great interest in cancer research due to their potential role as diagnostic and prognostic markers (Jamali *et al.* 2015, Falzone *et al.* 2018, 2019).

### *3.3.3 Piwi-Interacting RNAs*

Piwi-interacting RNAs (piRNAs) are made up of 26–31 nucleotides and transcribed from repetitive sequences in the genome (Bamezai *et al.* 2012). Few studies have investigated the role of piRNAs in OSCC. Some piRNAs have been correlated with HPV and smoking status (Firmino *et al.* 2016, Krishnan *et al.* 2017).

### *3.3.4 Circular RNAs*

Circular RNAs (circRNAs) are circles of ncRNAs whose 5’ and 3’ extremities are bound to create a covalently closed loop. circRNAs modulate gene expression through posttranscriptional control, miR sponging, or translational repression (Sun *et al.* 2016). Concerning their role in OSCC, many circRNAs

differentially expressed between OSCC and paired non-cancerous matched tissues have been recognized. Notably, up-regulation of circRNA_100290 with CDK6 was able to impact oral cancer proliferation (Chen *et al.* 2017).

### *3.3.5 Long Noncoding RNAs*

Currently, limited evidence is provided on the role of lncRNAs in OSCC. They are implied in all levels of gene modulation, epigenetic, transcriptional, or translational, mainly operating in basic cellular functions like proliferation, differentiation, apoptosis, and metastasis, which are all essential to cancer development and progression (Li *et al.* 2017). In OSCC, the most frequently upregulated lncRNAs include HOX Antisense Intergenic RNA (*HOTAIR*) (Min *et al.* 2017), FOXC1 Upstream Transcript, Noncoding (*FOXCUT*) (Kong *et al.* 2014), Metastasis-Associated Lung Adenocarcinoma Transcript-1 (*MALAT1*) (Chang *et al.* 2018), Urothelial Carcinoma- Associated 1 (*UCA 1*) (Yang *et al.* 2016), Taurine Upregulated Gene 1 (*TUG1*) (Liang *et al.* 2017), Long Noncoding RNA Colon Cancer-Associated Transcripts 1 and 2 (*CCAT1* and *CCAT2*) (Arunkumar *et al.* 2017), Ferritin Heavy Chain 1 Pseudogene 3 (*FTH1P3*) (Zhang 2017), H19: Imprinted Maternally Expressed Transcript (*H19*) (Zhang *et al.* 2017), and MIR31 Host Gene (MIR31HG/HIFCAR) (Shih *et al.* 2017), while the downregulated ones include Maternally Expressed Gene 3 (*MEG3*) (Jia *et al.* 2014).

# 4. PROGNOSTIC AND DIAGNOSTIC SIGNIFICANCE OF microRNAs IN ORAL CANCER: A BIOINFORMATIC ANALYSIS

## 4.1 Introduction

Despite being easily explored by clinicians, the oral cavity often presents malignancies in an advanced stage (Akbulut *et al.* 2011, Grafton-Clarke *et al*. 2019). In addition, no consensus has been reached regarding early biomarkers for oral cancer diagnosis. Salivary markers such as IL-6 and IL-8 have been suggested as oral cancer markers but they exhibit reduced sensitivity and specificity being over-expressed in many inflammatory conditions (St John *et al.* 2004, Sahibzada *et al.* 2017). Thus, the designation of novel and reliable biomarkers is strongly recommended to enhance both diagnostic and prognostic strategies in oral cancer. Notably, the development of advanced technologies for molecular and epigenetics investigations has permitted to collect numerous data that may be helpful for the identification of new cancer biomarkers (Falzone *et al.* 2018). Specifically, dysregulated miRs could be used for this purpose. Indeed, specific miRs have been specifically associated with tumor progression or with a worse prognosis (Hafsi *et al.* 2016, Falzone *et al.* 2019). In this context, numerous investigations have evaluated the data deriving from miRs microarray or sequencing profiling in oral cancer samples. However, the absence of a standardized integration approach between the different data matrices has determined contrasting data (Manikandan *et al.* 2016, Yan *et al.* 2017, Chamorro-Petronacci *et al.* 2019).

### *4.1.1 Aim*

To the best of our knowledge, no previous studies have simultaneously performed an integrated analysis on different oral cancer tissue miRs profiling datasets. Thus, the aim of this study was to identify a panel of miRs suitable as potential diagnostic and/or prognostic biomarkers for oral cancer. For this purpose, miRs expression datasets, contained in both the Gene Expression Omnibus DataSets (GEO DataSets) and The Cancer Genome Atlas (TCGA) Head and Neck Cancer (HNSC) were examined.

## 4.2 Material and Methods

### *4.2.1 miRs datasets selection*

The oral cancer datasets of microRNA profiling by array were identified by searching within the datasets included in the GEO DataSets portal available on NCBI (www.ncbi.nlm.nih.gov/geo/) (Barrett *et al.* 2013). To select the pertinent datasets, an advanced search was carried out applying the search terms "((“non coding RNA profiling by array” [DataSet Type]) and oral carcinoma)" and "Homo sapiens" [porgn: _txid9606]". From the list of datasets obtained, only those satisfying the following inclusion criteria were selected:

1) datasets concerning miRs expression related to oral cancer tissues, excluding hypopharynx, larynx, esophagus and tonsil;

2) datasets containing miRs expression levels of both tumor and normal tissue specimens;

3) datasets showing the miRs expression data of at least 30 samples (tumor + normal).

The exclusion criteria referred to datasets containing only data of tumor tissues; datasets including information about miRs referred to tumor or normal cell lines; and datasets with miRs expression levels obtained from serum samples.

Applying the abovementioned search criteria, 37 datasets of oral carcinoma microRNA profiling by array (updated up to December 2018) were identified. Nevertheless, 35 of these datasets did not meet the inclusion criteria. Thus, only two datasets were included to conduct the differential analyses (**Table 4.1**).

**Table 4.1** General characteristics of the included GEO DataSets.

| Series | Number normal | Number cancer | Samples | Platform | Reference | Number total |
|---|---|---|---|---|---|---|
| **GSE45238** | 40 | 40 | Fresh frozen tissues | GPL8179 Illumina Human v2 MicroRNA expression beadchip | Shiah *et al.* 2014 | 80 |
| **GSE31277** | 15 | 15 | Fresh frozen tissues | GPL9770 Illumina miR arrays version 1.0 | Severino *et al.* 2013 | 30 |

Besides the datasets listed in the GEO DataSets database, also the TCGA Head and Neck Cancer (HNSC) database was considered. Among the 25 datasets found in the TCGA HNSC database, the "Phenotype" and "miRNA mature strand expression RNAseq by Illumina Hiseq" HNSC datasets were downloaded for the analyses by means of UCSC Xena Browser (https://xenabrowser.net/) portal which reports all the HNSC molecular profiling data, derived by the TCGA consortium, including those of oral cancer. Specifically, the first dataset reported the clinical-pathological data of 604 samples (530 cancer patients and 74 normal) while the second the miRs expression profile of 529 samples. Given that the TCGA HNSC database also includes tumor samples concerning not only the oral

cavity but also other sites (i.e. oropharynx, hypopharynx, larynx, and tonsil), only the data referred to the alveolar ridge, base of tongue, buccal mucosa, floor of mouth, hard palate, lip, oral cavity, and oral tongue were examined. By verifying the inclusion criteria, the total of examined samples was limited to 399 and 351, respectively for two databases.

### *4.2.2 Analysis of miRs expression*

Data selected from the GEO DataSets and TCGA databases were subjected to different analyses.

#### *4.2.2.1 GEO datasets*

A first analysis was employed for both databases by incorporating the different GEO DataSets platform and matching the miRs expression levels of tumor specimens with normal to recognize new potential diagnostic biomarkers. Specifically, the data matrices of each dataset chosen from GEO DataSets were downloaded to detect the down-regulated or up-regulated miRs in oral cancer. The differential analysis between cancer and normal samples was conducted by GEO2R tool (Barrett *et al.* 2013). The fold change value (FC) referred to each miR was expressed as base-2 logarithm of FC (logFC) to uniform the data acquired from different microarray platforms. Subsequently, for each dataset only the differentially expressed miRs with a statistical significance $p<0.01$ were considered for further analysis. The altered miRs of the two included GEO DataSets platforms were then matched and only the miRs common to the two datasets and with a logFC value > ±1.5 were selected.

#### *4.2.2.2 TCGA HNSC dataset*

At the same time, other differential analyses of miRs expression levels between tumor vs normal samples and between high-grade vs low-grade tumors

of TCGA HNSC dataset were conducted to determine potential miRs with a prognostic power.

For the differential analyses, the samples were grouped based on presence or not of cancer [Cancer (348 samples) vs Control (51 samples)] and in accordance with the tumor stage [T3–T4 (319 samples) vs. T1–T2 (32 samples)]. When patients' stratification was performed, the fold change value collected by the differential analysis between the different clusters was calculated to identify the down-regulated and up-regulated miRs. The miRs expression levels were not available for some oral cancer patients (NA value) and consequently only the differentially expressed miRs with reported expression data for at minimum 50% of the patients and with *p*-value of $p<0.01$ were included for additional analysis. Moreover, regarding the differential analysis between tumor and control samples, only the 25 most up-regulated and down-regulated miRs were selected to provide more significant data; in relation to the differential analysis on tumor grade, all the differentially expressed miRs were taken into account. Lastly, the annotation of the TCGA HNSC miRs was conducted through miRBase V.22 (http://www.mirbase.org/) by transforming the miRNA IDs 'MIMAT00' in 'hsa-miR-'.

### *4.2.3 Analysis of the interaction between the selected miRs and the altered genes in oral cancer: COSMIC and mirDP analysis*

Once altered miRs were selected, an additional analysis was performed through different bioinformatics methods to determine the effective role of the selected miRs. First, the most de-regulated genes in oral cancer were identified using the data collected in the Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic). Then, for each of the COSMIC genes, the

specificity of miR-gene interaction was investigated through the bioinformatics prediction software miRs Data Integration Portal (mirDIP; http://ophid.utoronto.ca/mirDIP). More in detail, this software permits to merge the data referred to 26 distinct databases for miRs (including miRBase, microrna.org and DIANA microT-CDS v5). The level of interaction between the miRs and the targeted gene is categorized as very high, high, medium and low according to the integrated score calculated by the mirDIP algorithm that unifies the confidence scores from all available predictions data of the 26 databases (Shirdel *et al.* 2011; Tokar *et al.* 2018). Furthermore, the expression levels of the 10 interacting genes selected with COSMIC were subjected to the differential analysis of the gene expression data contained in the TCGA HNSC IlluminaHiSeq pancan normalized dataset.

### *4.2.4 Analysis of the interaction between the tumor/grade associated miRs and the altered genes in oral cancer: miRCancerdb analysis*

Besides the COSMIC analysis, a further correlation analysis was also carried out on the genes of TCGA HNSC dataset whose expression is up or down regulated by the selected tumor-associated miRs. For this purpose, the free R software miRCancerdb (https://mahshaaban.shinyapps.io/miRCancerdb/) was used, allowing the calculation of the correlation value applying the Pearson correlation coefficient (ρ) for each identified miR with different genes (Ahmed *et al.* 2018). In addition, the lists of genes collected for each miR were matched through the tool Draw Venn Diagrams of the Bioinformatics & Evolutionary Genomics (BEG) (https://bioinformatics.psb.ugent.be/webtools/Venn/) to detect the common genes to all miRs. Nevertheless, miRCancerdb employs only interaction data derived from the TargetScan database. Thus, the previously described mirDIP tool, that uses 26 different miR databases, was also applied to

define the levels of miRs-genes interaction. These analyses were conducted for the 11 miRs associated with oral cancer and for the 11 miRs that after OncoLnc analysis were correlated with both tumor grade and patients' overall survival (OS) and recurrence-free survival (RFS).

### *4.2.5 Kaplan-Meyer estimate of overall survival (OS) and recurrence-free survival (RFS) in patients with de-regulated tumor stage-related miRs*

The bioinformatics tool OncoLnc (http://www.oncolnc.org/) was used to assess the prognostic significance of the tumor stage-related miRs selected (Anaya 2016). OncoLnc is a tool that through the mortality data provided by TCGA datasets, including HNSC, is able obtain the Kaplan-Meier survival curves for each miR. The software recognizes which of the selected tumor stage-related miRs was associated with patients' overall survival (OS). The analysis was performed following the manufacturer' instructions that indicate to carry out the analysis between the expression levels of bottom quartile samples and top quartile samples. The survival curves were also calculated by using the TGCA survival data to corroborate the results obtained with the previous analysis. The TGCA survival data were downloaded only for the oral cancer (excluding hypopharynx, oropharynx, larynx, and tonsil) and analyzed with GraphPad v.6 (GraphPad Software, Inc, La Jolla, CA).

In addition, because no specific bioinformatics tools are currently available for the analysis of TCGA recurrence-free survival data, the RFS curves were calculated by using the TGCA HNSC progression data analyzed with a GraphPad survival curve sheet. More in detail, RFS was established from the time of the diagnosis to the patient progression, or to the end of follow-up, depending on which occurred first. The follow-up periods varied from patient to patient up

to a maximum of 5480 days. Of note, for some patients RFS data were not available.

### *4.2.6 Prediction pathway analysis, Gene Ontology (GO) and function of selected miRs*

A pathway prediction analysis was applied through tool DIANA-mirPath v.3 to specify the molecular pathways regulated by the tumor-associated selected miRs (Vlachos *et al.* 2015).

Then, the pathways enrichment analysis of the lists of genes obtained from the miRCancerdb and DIANA-mirPath v.3 was performed, allowing determination of the functional role of the selected miRs. In this regard, GO PANTHER version 14.0 (http://pantherdb.org/) and STRING version 11.0 (https://string-db.org/) software were adopted (Mi *et al.* 2019, Szklarczyk *et al*. 2019). This twofold approach was adopted to ensure a more comprehensive analysis. Indeed, STRING database refers to functional classification systems including GO, Pfam and KEGG and thus allow more robust results than those acquired from the GO PANTHER analysis. Of note, the data acquired with these biological functional prediction analyses are already uniformed with data assumed as reference or negative control.

The DIANA-mirPath, GO PANTHER and STRING analyses were performed for the 11 selected miRs correlated with oral cancer and for the 11 miRs that after OncoLnc analysis were associated with both tumor grade and patients' OS and RFS.

### *4.2.7 Statistical analysis*

Different statistical tests were used through the bioinformatics analysis. The miRs expression data obtained from the GEO DataSets were already

normalized by the GEO2R software. As regards the fold change values of TCGA HNSC miR expression levels, they were calculated by using differential analysis. The significant differentially expressed miRs of the TCGA dataset were identified through the Student's t-test. Regarding GEO DataSets, the GEO2R software calculated the p-values for each of them. GraphPad survival sheet and log-rank non-parametric test were applied for the Kaplan-Meier analyses. The significance level (*p*-value) was set to 0.05 and 0.01.

## 4.3 Results

### *4.3.1 Selection of oral cancer associated-miRs with a diagnostic role*

#### *4.3.1.1 GEO datasets*

The differential analysis conducted with GEO2R on the two GEO DataSets databases resulted in two lists of altered miRs in oral tumors in comparison with normal controls. By matching these two lists, 28 miRs differentially expressed were detected in the tumor tissues, 12 of which were up-regulated and 16 down-regulated (**Table 4.2**).

#### *4.3.1.2 TCGA HNSC datasets*

The analysis of the expression data regarding miRs present in the TCGA HNSC dataset resulted in a list of 514 de-regulated miRs associated with oral cancer ($p<0.01$; **Table S4.1**). Interestingly, 21 of the 28 miRs selected with the GEO DataSets analysis were also represented in this list of 514 miRs, thus corroborating the validity of results obtained. To select the most relevant miRs correlated with oral cancer, the 25 most up-regulated and the 25 most down-regulated miRs were chosen from the list of 514 miRNAs (**Table 4.3**).

**Table 4.2** Deregulated (up- and down-) miRs of GEODataSets in tumor samples vs normal.

| miR ID | GSE45238 | | GSE31277 | |
|---|---|---|---|---|
| | Fold change | p-value* | Fold change | p-value* |
| **Up-regulated miRs** | | | | |
| hsa-miR-196a-5p | 8.096 | 9.45E-12 | 8.132 | 1.42E-06 |
| hsa-miR-503-5p | 5.010 | 4.83E-21 | 2.622 | 4.69E-04 |
| hsa-miR-7-5p | 3.505 | 9.41E-20 | 2.297 | 5.00E-04 |
| hsa-miR-542-5p | 3.348 | 9.21E-12 | 2.700 | 1.10E-04 |
| hsa-miR-142-5p | 3.323 | 3.98E-08 | 2.633 | 2.12E-03 |
| hsa-miR-19a-3p | 3.068 | 3.81E-07 | 2.910 | 4.75E-04 |
| hsa-miR-18a-5p | 2.646 | 2.34E-10 | 1.554 | 2.66E-03 |
| hsa-miR-19b-3p | 2.179 | 1.28E-05 | 2.415 | 7.73E-04 |
| hsa-miR-32-5p | 1.997 | 1.76E-05 | 3.874 | 3.28E-05 |
| hsa-miR-196b-5p | 1.791 | 2.05E-08 | 1.874 | 2.00E-04 |
| hsa-miR-33b-5p | 1.581 | 9.26E-04 | 2.541 | 2.00E-03 |
| hsa-miR-34b-3p | 1.558 | 1.95E-04 | 2.079 | 1.13E-03 |
| **Down-regulated miRs** | | | | |
| hsa-miR-195-5p | -1.778 | 1.25E-12 | -1.620 | 1.71E-06 |
| hsa-miR-378a-5p | -1.799 | 9.47E-12 | -2.194 | 4.45E-03 |
| hsa-miR-363-3p | -1.869 | 1.56E-05 | -1.951 | 4.16E-05 |
| hsa-miR-100-5p | -1.883 | 8.04E-14 | -2.199 | 1.19E-04 |
| hsa-miR-328-5p | -2.471 | 1.18E-08 | -1.599 | 2.32E-03 |
| hsa-miR-99a-5p | -2.732 | 4.83E-16 | -2.441 | 7.82E-05 |
| hsa-miR-218-5p | -3.021 | 1.08E-10 | -1.853 | 1.72E-04 |
| hsa-miR-432-5p | -3.155 | 1.55E-13 | -1.718 | 3.14E-03 |
| hsa-miR-379-5p | -3.513 | 1.83E-11 | -2.345 | 9.63E-04 |
| hsa-miR-154-5p | -4.021 | 4.01E-13 | -1.826 | 2.00E-03 |
| hsa-miR-133a-3p | -4.202 | 6.37E-09 | -3.446 | 8.47E-03 |
| hsa-miR-487b-5p | -4.366 | 6.96E-15 | -1.899 | 9.71E-03 |
| hsa-miR-135a-5p | -4.910 | 1.11E-14 | -3.324 | 1.90E-03 |
| hsa-miR-411-5p | -5.574 | 3.25E-16 | -2.542 | 6.18E-03 |
| hsa-miR-1-3p | -9.783 | 3.47E-09 | -5.786 | 2.16E-03 |
| hsa-miR-375 | -16.589 | 1.95E-17 | -3.198 | 5.12E-04 |

**p-values were automatically calculated through the GEO2R software by applying Student's t-test*

**Table 4.3** Deregulated (up- and down-) miRs of TCGA dataset in tumor samples vs normal. In bold, the miRs shared with GEODatasets anlysis.

| miRNA ID | miRNA name | FC Cancer vs Normal | p-value* |
|---|---|---|---|
| **Up-regulated** | | | |
| **MIMAT0000226** | **hsa-miR-196a-5p** | **12.145** | **3.12E-19** |
| **MIMAT0001080** | **hsa-miR-196b-5p** | **11.639** | **5.43E-20** |
| MIMAT0000267 | hsa-miR-210-3p | 9.733 | 1.18E-09 |
| MIMAT0000089 | hsa-miR-31-5p | 7.684 | 8.42E-12 |
| MIMAT0004784 | hsa-miR-455-3p | 7.165 | 9.21E-18 |
| MIMAT0005923 | hsa-miR-1269a | 5.899 | 1.99E-11 |
| MIMAT0000102 | hsa-miR-105-5p | 5.510 | 9.64E-13 |
| MIMAT0004504 | hsa-miR-31-3p | 5.298 | 1.59E-09 |
| MIMAT0003882 | hsa-miR-767-5p | 5.294 | 5.40E-13 |
| MIMAT0000281 | hsa-miR-224-5p | 4.789 | 5.39E-11 |
| **MIMAT0002874** | **hsa-miR-503-5p** | **4.044** | **3.86E-19** |
| MIMAT0002819 | hsa-miR-193b-3p | 3.407 | 8.17E-15 |
| MIMAT0005951 | hsa-miR-1307-3p | 3.395 | 1.14E-11 |
| MIMAT0000076 | hsa-miR-21-5p | 3.209 | 3.05E-10 |
| MIMAT0000266 | hsa-miR-205-5p | 3.040 | 1.64E-05 |
| MIMAT0016895 | hsa-miR-2355-5p | 3.023 | 6.22E-14 |
| MIMAT0004987 | hsa-miR-944 | 3.020 | 7.56E-07 |
| MIMAT0005797 | hsa-miR-1301-3p | 2.902 | 6.39E-17 |
| MIMAT0000761 | hsa-miR-324-5p | 2.878 | 7.41E-12 |
| MIMAT0000758 | hsa-miR-135b-5p | 2.859 | 4.08E-08 |
| MIMAT0001341 | hsa-miR-424-5p | 2.856 | 4.57E-13 |
| **MIMAT0000072** | **hsa-miR-18a-5p** | **2.829** | **8.10E-10** |
| MIMAT0001545 | hsa-miR-450a-5p | 2.828 | 1.20E-15 |
| MIMAT0000688 | hsa-miR-301a-3p | 2.807 | 5.32E-13 |
| MIMAT0003150 | hsa-miR-455-5p | 2.799 | 3.50E-12 |
| **Down-regulated** | | | |
| MIMAT0002870 | hsa-miR-499a-5p | -3.296 | 3.76E-05 |
| **MIMAT0000733** | **hsa-miR-379-5p** | **-3.298** | **1.29E-10** |
| MIMAT0002890 | hsa-miR-299-5p | -3.504 | 8.97E-07 |
| **MIMAT0000461** | **hsa-miR-195-5p** | **-3.510** | **7.79E-14** |
| MIMAT0022721 | hsa-miR-1247-3p | -3.553 | 3.40E-07 |
| MIMAT0016847 | hsa-miR-378c | -3.670 | 4.61E-08 |
| MIMAT0002171 | hsa-miR-410-3p | -3.684 | 9.33E-12 |
| MIMAT0004603 | hsa-miR-125b-2-3p | -3.694 | 1.52E-18 |
| MIMAT0004606 | hsa-miR-136-3p | -3.797 | 1.08E-12 |
| MIMAT0004550 | hsa-miR-30c-2-3p | -3.881 | 1.03E-12 |
| MIMAT0004552 | hsa-miR-139-3p | -3.937 | 3.02E-14 |
| MIMAT0000099 | hsa-miR-101-3p | -4.017 | 3.64E-23 |
| MIMAT0000087 | hsa-miR-30a-5p | -4.132 | 6.93E-14 |
| **MIMAT0003329** | **hsa-miR-411-5p** | **-4.160** | **2.03E-10** |
| MIMAT0000265 | hsa-miR-204-5p | -4.519 | 1.28E-17 |
| MIMAT0000681 | hsa-miR-29c-3p | -4.539 | 5.24E-17 |
| MIMAT0000064 | hsa-let-7c-5p | -4.674 | 3.68E-22 |
| MIMAT0000462 | hsa-miR-206 | -5.228 | 4.62E-03 |
| MIMAT0000736 | hsa-miR-381-3p | -5.293 | 5.06E-08 |
| MIMAT0000770 | hsa-miR-133b | -5.580 | 3.66E-04 |
| MIMAT0000088 | hsa-miR-30a-3p | -5.696 | 2.66E-13 |
| **MIMAT0000097** | **hsa-miR-99a-5p** | **-5.746** | **1.85E-27** |
| **MIMAT0000427** | **hsa-miR-133a-3p** | **-7.055** | **2.93E-04** |
| **MIMAT0000416** | **hsa-miR-1-3p** | **-10.663** | **8.80E-06** |
| **MIMAT0000728** | **hsa-miR-375-3p** | **-18.183** | **1.33E-11** |

**p-values were obtained with Student's t-test*

#### *4.3.1.3 Common-shared diagnostic miRs in GEO and TCGA datasets*

In **Table 4.3**, the miRs contained both in GEO and TCGA DataSets are represented in bold. It is reasonable to suppose that these common-shared miRs are the most significant implicated in cancer transformation. Moreover, most of these miRs provided the highest levels of up-regulation (miR-196a-5p and miR-196b-5p) and down-regulation (miR-99a-5p, miR-133a-3p, miR-1-3p and miR-375-3p).

In brief, the differential analyses conducted on GEO and TCGA datasets to identify miRs differentially expressed between tumor and control samples, demonstrated that 11 miRs, of which 4 up-regulated and 7 down-regulated, were strictly associated with oral cancer (**Table 4.4**).

As reported in **Table 4.4**, the up-regulated miR-196a-5p and the two down-regulated miRs miR-1-3p and miR-375-3p exhibited the higher levels of over-expression or down-regulation in all three datasets.

The subsequent prediction analyses of target genes and altered molecular pathways were applied on the 11 miRNAs reported in **Table 4.4.**

### *4.3.2 Levels of interaction between the 11-selected diagnostic miRs and altered genes in oral cancer: COSMIC and mirDP analysis*

The majority of the altered genes in oral tumors and miR-gene interaction specificity were determined by using COSMIC and mirDIP tools, respectively.

First, through COSMIC analysis, the 10 most recurrent mutations and gene alterations associated with oral cancer were recognized: *TP53* genes (43%), *FAT1* (28%), *CASP8* (23%), *TERT* (22%), *NOTCH1* (20%), *CDKN2A* (16%), *HRAS* (10%), *KMT2D* (10%), *FGFR3* (8%) and *PIK3CA* (8%).

**Table 4.4** General overview of miRs differentially expressed in "Cancer vs Normal" samples present both in GEO and TCGA HNSC datasets.

| | GEO DataSets | | | | TCGA HSNC Dataset | |
|---|---|---|---|---|---|---|
| | GSE45238 | | GSE31277 | | | |
| **miRs** | **FC Cancer vs Normal** | **p-value*** | **FC Cancer vs Normal** | **p-value*** | **FC Cancer vs Normal** | **p-value**** |
| **Up-regulated** | | | | | | |
| hsa-miR-196a-5p | 8.096 | 9.45E-12 | 8.132 | 1.42E-06 | 12.145 | 3.12E-19 |
| hsa-miR-196b-5p | 1.791 | 2.05E-08 | 1.874 | 2.00E-04 | 11.639 | 5.43E-20 |
| hsa-miR-503-5p | 5.010 | 4.83E-21 | 2.622 | 4.69E-04 | 4.044 | 3.86E-19 |
| hsa-miR-18a-5p | 2.646 | 2.34E-10 | 1.554 | 2.66E-03 | 2.829 | 8.10E-10 |
| **Down-regulated** | | | | | | |
| hsa-miR-379-5p | -3.513 | 1.83E-11 | -2.345 | 9.63E-04 | -3.298 | 1.29E-10 |
| hsa-miR-195-5p | -1.778 | 1.25E-12 | -1.620 | 1.71E-06 | -3.510 | 7.79E-14 |
| hsa-miR-411-5p | -5.574 | 3.25E-16 | -2.542 | 6.18E-03 | -4.160 | 2.03E-10 |
| hsa-miR-99a-5p | -2.732 | 4.83E-16 | -2.441 | 7.82E-05 | -5.746 | 1.85E-27 |
| hsa-miR-133a-3p | -4.202 | 6.37E-09 | -3.446 | 8.47E-03 | -7.055 | 2.93E-04 |
| hsa-miR-1-3p | -9.783 | 3.47E-09 | -5.786 | 2.16E-03 | -10.663 | 8.80E-06 |
| hsa-miR-375-3p | -16.589 | 1.95E-17 | -3.198 | 5.12E-04 | -18.183 | 1.33E-11 |

**p-values obtained with GEO2R software; **p-values calculated through the Student's t-test*

Then, by using mirDIP tool, the interaction levels between the 11-oral cancer-associated miRs and the genes selected with COSMIC have been detected (**Table S4.2**).

For the 10 interacting genes, expression levels were also analyzed using the TCGA HNSC IlluminaHiSeq pancan normalized dataset (**Table S4.3**). This analysis proved that all the selected miRs interacted with the frequently mutated genes in oral cancer. Specifically, most interactions exhibited a medium-high specificity confirming the strong association between altered miRs in oral cancer and the genes implicated in pivotal functional pathways (**Figure 4.1**). Of note, the analysis of the TCGA HNSC IlluminaHiSeq pancan normalized dataset demonstrated that only 6 of the 10 genes (*TP53*, *FAT1*, *CASP8*, *TERT*, *CDKN2A,* and *PIK3CA*) were significantly de-regulated in oral cancers (**Table S4.3**).

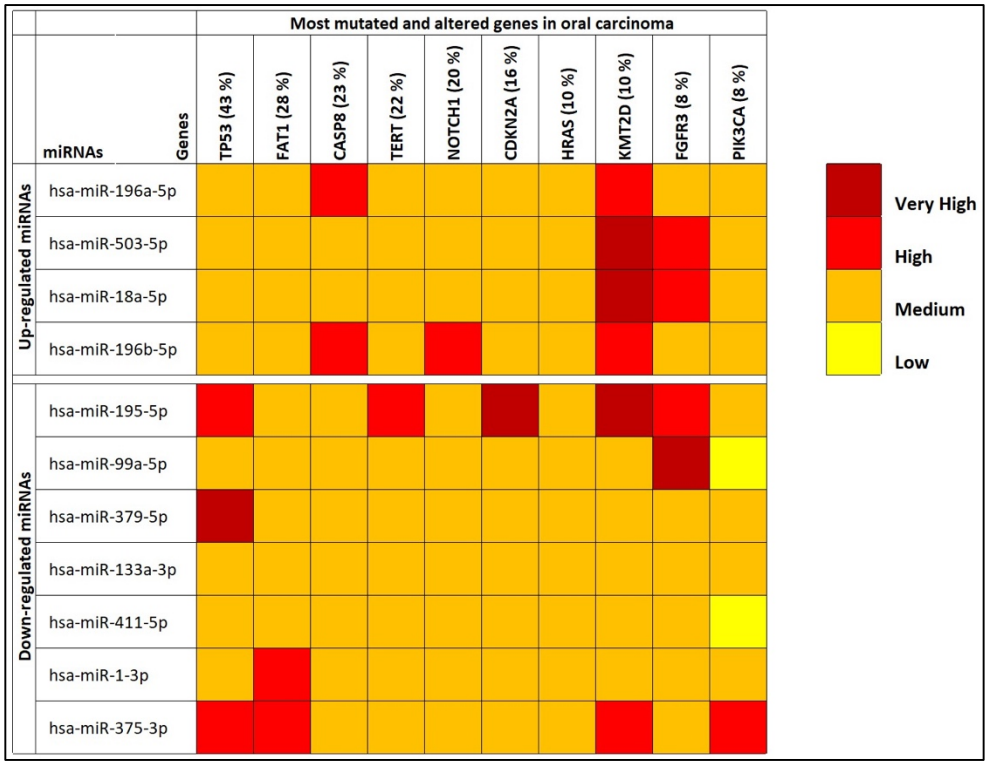


**Figure 4.1** mirDIP analysis of interaction levels between the 11 miRs and the principal mutated genes in oral tumors.

Interestingly, all up-regulated miRs could target the *KMT2D* gene by decreasing its expression levels. Interestingly, *KMT2D* is a tumor suppressor gene, therefore its down-regulation promoted by the up-regulated miRs activate cellular neoplastic transformation.

Concerning the miRs, the 11 selected miRs exhibited medium levels of interaction (represented in orange) with the target genes, with the down regulated hsa-miR-195-5p and has-miR-375-3p presenting the maximum interaction levels with the corresponding genes (**Figure 4.1**).

In addition, *FAT1*, *CASP8*, *TERT*, *CDKN2A,* and *PIK3CA* genes were significantly up-regulated in the tumor specimens, while *TP53* was significantly down-regulated.

### *4.3.3 Levels of interaction between the 11-selected diagnostic miRs and the altered genes in oral cancer: miRCancerdB analysis*

The correlation value of each miR with different genes was acquired through the bioinformatics tool miRCancerdb. Specifically, for each miR a list of miRs-correlated genes, varying from 4493 to 9042, was gained. Then, these lists were matched resulting in 121 common-shared genes. Nevertheless, only the genes shared by the 11 miRs and presenting in the first quartile of the genes most positively and negatively correlated with each miR were considered (**Figure 4.2**). This selection revealed the correlation levels of 105 different genes (**Figure 4.2A**).

In **Figure 4.2A**, the down-regulated miR-133a-3p and miR-1-3p exhibited the highest positive correlation levels while the up-regulated miR-18a-5p showed the lowest negative correlation level. Furthermore, the *FYCO1*, *SORBS1* and *GPD1L* genes exhibited a strong, positive correlation with the selected miRs; conversely, *ASA1*, *NFIC* and *SECISBP2L* genes resulted in the lowest level of correlation.

To confirm the correlation levels observed, the mirDIP tool was also applied (**Figure 4.2B**). For 8 genes (*KIAA1370*, *CHP*, *WDR67*, *ZNF642*, *LASS5*, *ORC6L*, *C20orf20*, *C1orf135*), no interactions were detected with the selected miRs. The up-regulated miR-195-5p, miR-503-5p, and miR-18a-5p as well as the down-regulated miR-375-3p exhibited the highest interaction levels with the 105 genes. As concerns for the genes involved, *CPEB3*, *CPEB4*, *MAGI1*, *PHACTR2*, *PDLIM5*, *NFIC*, *SLMAP* and *SECISBP2L* strongly interacted with the 11 selected miRs (**Figure 4.2B**).

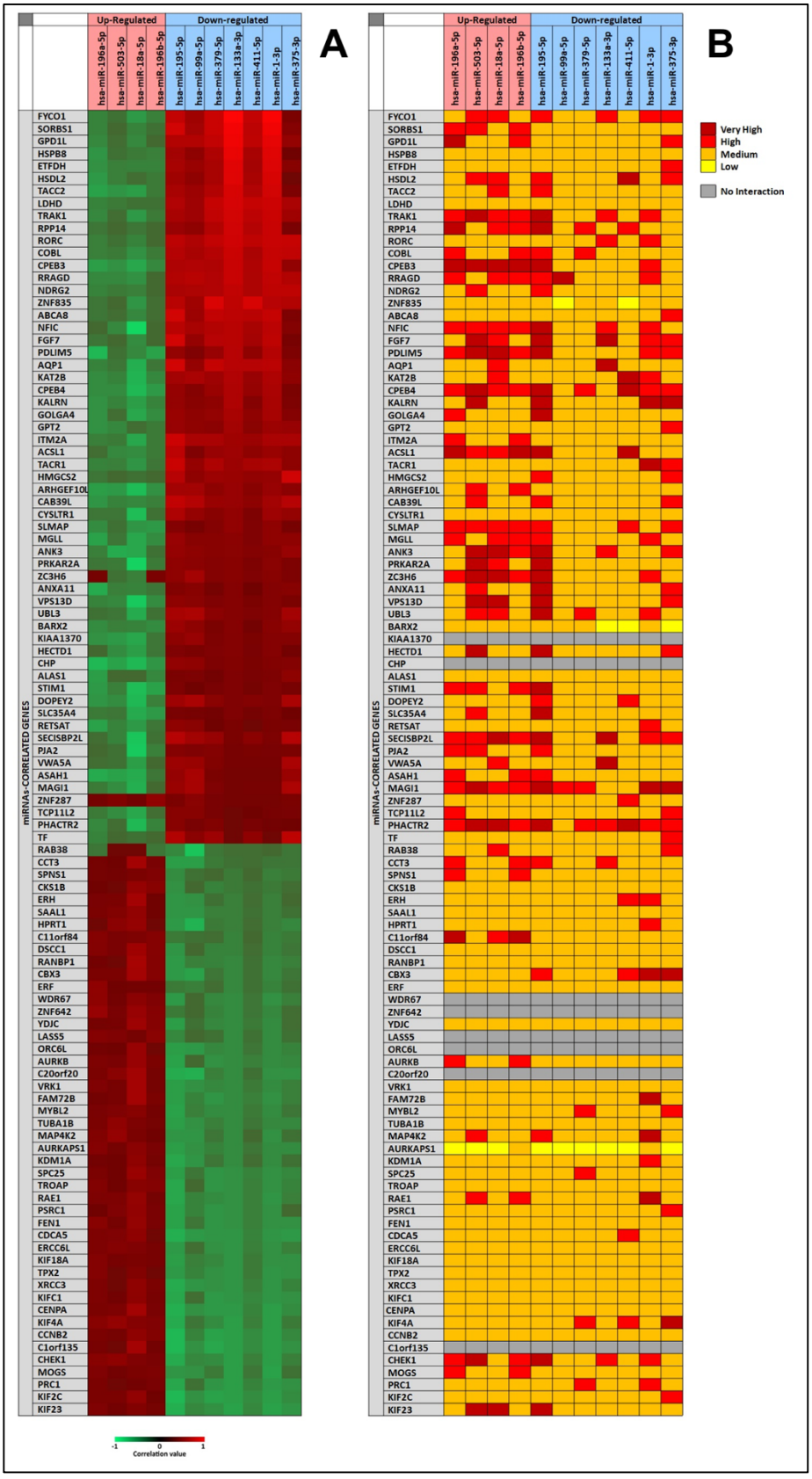


**Figure 4.2 A, B A)** miRCancerdb analysis of genes positively and negatively correlated with the 11 miRs; **B)** mirDIP analysis of interaction levels between the miRs and the altered genes.

### *4.3.4 Functional role of the 11-selected diagnostic miRs: Pathway and GO Enrichment Analyses*

Concerning the pathway prediction analysis, all the 11 selected miRs were inserted into the bioinformatics prediction tool DIANA-mirPath. The analysis revealed that the miR-503-5p, miR-133a-3p, and miR-1-3p were not correlated with pathways and targeted genes. On the other hand, the other miRs were able to modify 48 distinct pathways and more 2100 genes. Specifically, the pathways implicated in carcinogenesis were 22 and the modulated genes 345 (**Table 4.5**). The pathways strictly controlled by the 11 miRs were: "Pathways in cancer (hsa05200)", "Cell cycle (hsa04110)", several signal transduction pathways including "FoxO signaling pathway (hsa04068)", "p53 signaling pathway (hsa04115)" and "Hippo signaling pathways (hsa04390)".

Inside these pathways, *MAPK1, CCND1, AKT3, PIK3CA, PIK3CB, NRAS, BRAF, CDK4, CDKN1A,* and *E2F2* genes resulted frequently de-regulated by the selected miRs. As is well known, the alteration of these genes is able to promote cancer development and progression.

To validate the results obtained, gene enrichment analyses were conducted on the 105 miRCancerdb genes by applying both GO PANTHER and STRING software. The analyses provided overlapping results with respect to the three ontological categories: "biological process", "molecular function" and "cellular component" (**Figure 4.3**).

Regarding the "biological process" category, the most miRs-modulated genes were implicated in the regulation of biological (29.9% and 78.7%, GO PANTHER and STRING, respectively) and cellular (17.8% and 75.6%, GO PANTHER and STRING, respectively) processes (**Figure 4.3 A, D**).

**Table 4.5** Pathways implicated in carcinogenesis and targeted by the 11-selected miRs.

| N. | KEGG pathway | Up-regulated miRs | | | Down-regulated miRs | | |
|---|---|---|---|---|---|---|---|
| | | p-value* | genes | miRs | p-value* | genes | miRs |
| **1** | Bladder cancer (hsa05219) | 2.25E-03 | 14 | 3 | 2.78E-03 | 19 | 5 |
| **2** | Cell cycle (hsa04110) | 1.11E-02 | 27 | 3 | 5.48E-03 | 43 | 6 |
| **3** | Central carbon metabolism in cancer (hsa05230) | / | / | / | 4.59E-02 | 20 | 5 |
| **4** | Chronic myeloid leukemia (hsa05220) | 3.61E-04 | 22 | 3 | 1.99E-02 | 25 | 5 |
| **5** | Colorectal cancer (hsa05210) | 7.53E-05 | 18 | 3 | / | / | / |
| **6** | FoxO signaling pathway (hsa04068) | 7.64E-03 | 28 | 3 | 4.50E-03 | 44 | 6 |
| **7** | Glioma (hsa05214) | 2.56E-03 | 16 | 3 | 3.70E-03 | 23 | 5 |
| **8** | Hippo signaling pathway (hsa04390) | 1.74E-11 | 41 | 3 | 4.22E-08 | 51 | 6 |
| **9** | Melanoma (hsa05218) | 1.48E-02 | 15 | 3 | / | / | / |
| **10** | mTOR signaling pathway (hsa04150) | / | / | / | 1.82E-02 | 22 | 5 |
| **11** | Non-small cell lung cancer (hsa05223) | 2.54E-02 | 14 | 3 | / | / | / |
| **12** | p53 signaling pathway (hsa04115) | 1.84E-03 | 19 | 3 | 6.53E-04 | 28 | 6 |
| **13** | Pancreatic cancer (hsa05212) | 2.90E-02 | 17 | 3 | 4.79E-02 | 23 | 5 |
| **14** | Pathways in cancer (hsa05200) | 1.33E-03 | 62 | 3 | 1.68E-04 | 111 | 6 |
| **15** | Prostate cancer (hsa05215) | 3.73E-02 | 19 | 3 | 3.83E-03 | 33 | 6 |
| **16** | Proteoglycans in cancer (hsa05205) | 2.13E-04 | 35 | 3 | 1.11E-12 | 73 | 6 |
| **17** | Renal cell carcinoma (hsa05211) | / | / | / | 1.65E-02 | 23 | 6 |
| **18** | Small cell lung cancer (hsa05222) | 2.34E-02 | 19 | 3 | 1.65E-02 | 29 | 5 |
| **19** | TGF-beta signaling pathway (hsa04350) | 8.01E-06 | 19 | 3 | 6.45E-03 | 26 | 6 |
| **20** | Thyroid cancer (hsa05216) | 3.68E-02 | 7 | 3 | / | / | / |
| **21** | TNF signaling pathway (hsa04668) | / | / | / | 1.88E-02 | 36 | 6 |
| **22** | Viral carcinogenesis (hsa05203) | 1.53E-02 | 35 | 3 | 3.77E-06 | 65 | 6 |

** p-values were obtained with the DIANA-mirPath by automatically using the Fisher's Exact Test*

As concerns for the "molecular function" category, all the genes were principally involved in the binding and the catalytic activities according to the GO PANTHER (**Figure 4.3B**) analysis and in protein binding, cyclic compounds and nucleotides binding as for STRING analysis (**Figure 4.3E**).

Finally, concerning the "cellular component" category, the greater part of the genes encoded components of the cell (39.3% and 93.3% for GO PANTHER

and STRING, respectively) and organelles (23.1% and 84.8% for GO PANTHER and STRING, respectively) (**Figure 4.3 C, F**).

The same GO enrichment analyses were conducted on the 345 genes identified by DIANA-mirPath showing comparable results to those illustrated above (**Figure 4.4 A-F**).

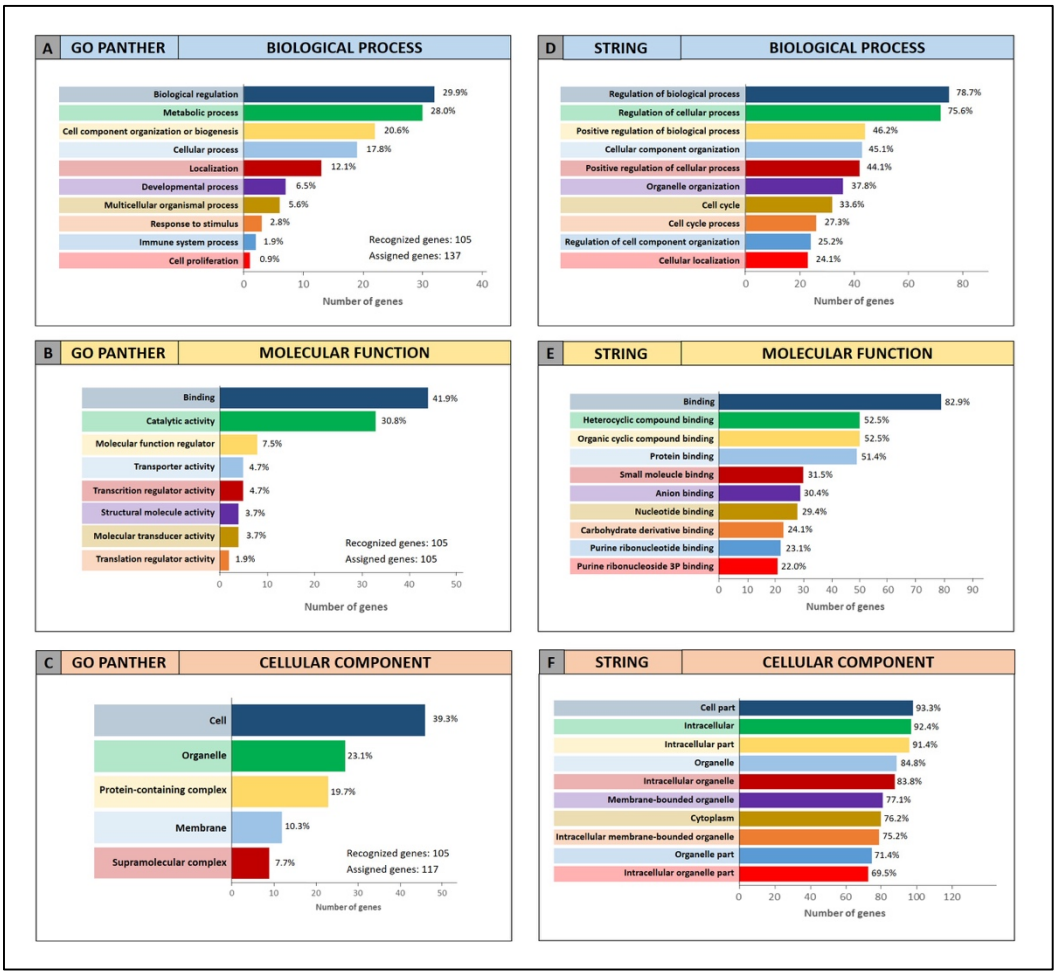


**Figure 4.3 A-F** Gene Ontology enrichment of the 105 genes selected with miRCancerdb. **A)** GO PANTHER and **D)** STRING analyses referred to the "biological process" category; **B)** GO PANTHER and **E)** STRING analyses related to the "molecular function" category; **C)** GO PANTHER and **F)** STRING analyses concerning the "cellular component" category.

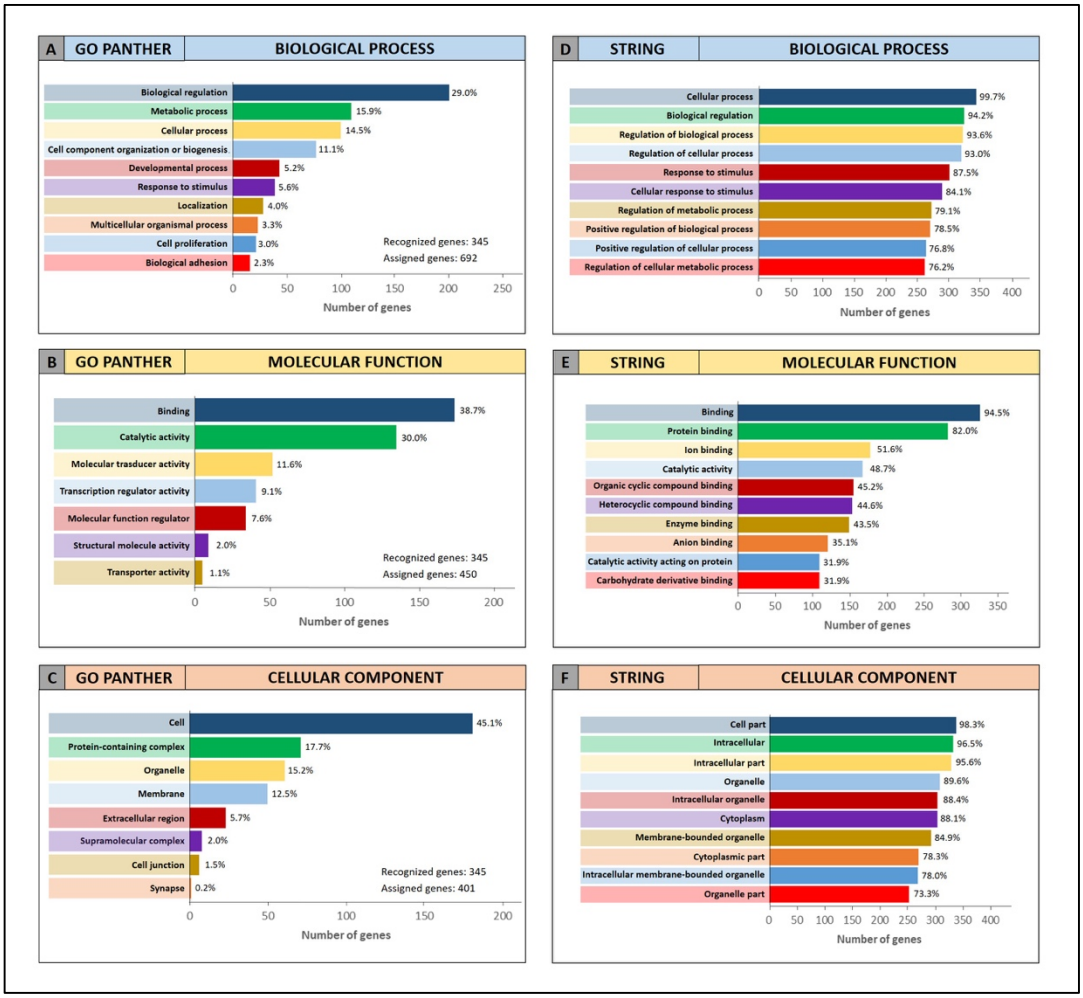


**Figure 4.4 A-F** Gene Ontology enrichment of the 345 genes selected with DIANA-mirPath. **A)** GO PANTHER and **D)** STRING analyses referred to the "biological process" category; **B)** GO PANTHER and **E)** STRING analyses related to the "molecular function" category; **C)** GO PANTHER and **F)** STRING analyses concerning the "cellular component" category.

### *4.3.5 Selection of the oral cancer associated-miRs with a prognostic role*

The same differential analysis performed to identify miRs with a potential diagnostic role was also applied to samples with different tumor grade to detect eventual miRs with a prognostic significance. This second differential analysis revealed that 36 miRs were de-regulated (31 down-regulated and 5 up-regulated) in high-grade samples compared to low-grade ($p$<0.01; **Table 4.6**).

Interestingly, among the 31 down-regulated miRs, three were shared with the miRs differentially expressed between tumor and normal samples (i.e. miR-139-3p, miR-142-5p, and miR-29c-3p) while the down-regulated miR-133a-3p

was in common with the list of 11 miRs collected from the comparison between GEO DataSets and TCGA analyses. These data suggested that the above mentioned miRs may have both diagnostic and prognostic significance in oral cancers.

**Table 4.6** TCGA analysis of deregulated (up- and down-) miRs in high-grade tumors compared with low-grade.

| miR ID | miR name | FC High-Grade vs Low-Grade | p-value** |
|---|---|---|---|
| **Up-regulated** | | | |
| MIMAT0001536 | hsa-miR-429 | 1.279 | 3.20E-03 |
| MIMAT0003233 | hsa-miR-551b-3p | 1.205 | 1.31E-03 |
| MIMAT0004697 | hsa-miR-151a-5p | 1.172 | 3.78E-03 |
| MIMAT0003246 | hsa-miR-581 | 1.078 | 3.88E-03 |
| MIMAT0019931 | hsa-miR-4775 | 1.064 | 1.31E-03 |
| **Down-regulated** | | | |
| MIMAT0004594 | hsa-miR-132-5p | -1.141 | 4.88E-03 |
| MIMAT0000727 | hsa-miR-374a-5p | -1.148 | 7.65E-03 |
| MIMAT0022272 | hsa-miR-664b-3p | -1.159 | 9.42E-04 |
| MIMAT0000415 | hsa-let-7i-5p | -1.180 | 2.57E-03 |
| MIMAT0003338 | hsa-miR-660-5p | -1.202 | 5.19E-03 |
| MIMAT0004775 | hsa-miR-502-3p | -1.206 | 4.61E-05 |
| MIMAT0000082 | hsa-miR-26a-5p | -1.209 | 4.96E-03 |
| MIMAT0004694 | hsa-miR-342-5p | -1.213 | 4.45E-03 |
| MIMAT0004766 | hsa-miR-146b-3p | -1.222 | 7.84E-03 |
| MIMAT0025849 | hsa-miR-6718-5p | -1.223 | 3.61E-04 |
| MIMAT0004682 | hsa-miR-361-3p | -1.224 | 8.00E-04 |
| MIMAT0004597 | hsa-miR-140-3p | -1.232 | 5.38E-05 |
| MIMAT0004673 | hsa-miR-29c-5p | -1.234 | 1.88E-03 |
| MIMAT0002808 | hsa-miR-511-5p | -1.246 | 9.53E-03 |
| MIMAT0000250 | hsa-miR-139-5p | -1.248 | 2.75E-03 |
| MIMAT0004585 | hsa-let-7i-3p | -1.251 | 7.09E-03 |
| MIMAT0019071 | hsa-miR-4532 | -1.256 | 3.56E-03 |
| MIMAT0019927 | hsa-miR-4772-3p | -1.258 | 6.01E-03 |
| MIMAT0000258 | hsa-miR-181c-5p | -1.267 | 1.17E-03 |
| MIMAT0004570 | hsa-miR-223-5p | -1.285 | 7.97E-03 |
| MIMAT0000086 | hsa-miR-29a-3p | -1.290 | 1.94E-03 |
| MIMAT0004552 | **hsa-miR-139-3p** | **-1.314** | **2.04E-03** |
| MIMAT0000433 | **hsa-miR-142-5p** | **-1.329** | **3.76E-03** |
| MIMAT0000646 | hsa-miR-155-5p | -1.349 | 5.08E-03 |
| MIMAT0000274 | hsa-miR-217-5p | -1.354 | 6.83E-03 |
| MIMAT0000449 | hsa-miR-146a-5p | -1.375 | 1.56E-03 |
| MIMAT0000280 | hsa-miR-223-3p | -1.397 | 1.40E-03 |
| MIMAT0000681 | **hsa-miR-29c-3p** | **-1.430** | **1.90E-03** |
| MIMAT0000451 | hsa-miR-150-5p | -1.644 | 3.98E-04 |
| MIMAT0000427 | **hsa-miR-133a-3p** | **-2.168** | **6.39E-03** |
| MIMAT0000462 | hsa-miR-206 | -3.070 | 1.29E-03 |

*In bold, miRs identified in the differential analysis "Cancer vs Normal" conducted on the TCGA and GEO Datasets; *miR present in the list of the 11-selected miRs; **p-values were obtained with Student's t-test*

### *4.3.6 Prognostic value of oral cancer stage-related miRs*

The OncoLnc analysis conducted on the 36 differently expressed miRs in high-grade oral cancers clarified the effective prognostic significance of each miR in terms of patients' overall survival (**Figure 4.5 A,B**).

Nine of 36 miRs analyzed were statistically correlated with patients' OS (log-rank test, $p<0.05$) and all were down-regulated (i. e. miR-181c-5p, miR-342-5p, miR-361-3p, miR-29c-5p, miR-142-5p, miR-146a-5p, miR-150-5p, miR-146b-3p, and miR-206). Interestingly, two of these miRs, miR-146b-3p and miR-206, have reported results of dubious interpretation: despite being down-regulated in high-grade tumors, these miRs were not linked with a worse OS but to a favorable prognosis (**Figure 4.5B**). To corroborate the results acquired with OncoLnc, the OS curves were also calculated by using GraphPad v.6 and examining the TGCA HNSC survival data previously downloaded from the UCSC Xena Browser. This analysis obtained similar results to those provided by OncoLnc.

The TCGA HNSC data were also employed to detect miRs able to predict the risk of oral cancer recurrence: among the 36-tumor stage-related miRs, the up-regulated miR-581 and the down-regulated miR-let-7i-3p were statistically correlated with the patients' recurrence-free survival (RFS). Surprisingly, the up-regulated miR-581 was not associated with a worse prognosis, but with a minor RFS (**Figure 4.6**).

Other five miRs (i.e. miR-151a-5p, miR-6718-5p, miR-660-5p, miR-4772-3p, and miR-217-5p), revealed a weak correlation with RFS when de-regulated even if no statistical significance was reached.

In summary, these analyses recognized 11 miRs significantly associated with both tumor grade and patients' OS and RFS, of which only seven were linked with patients' OS and one miR with RFS, respectively.

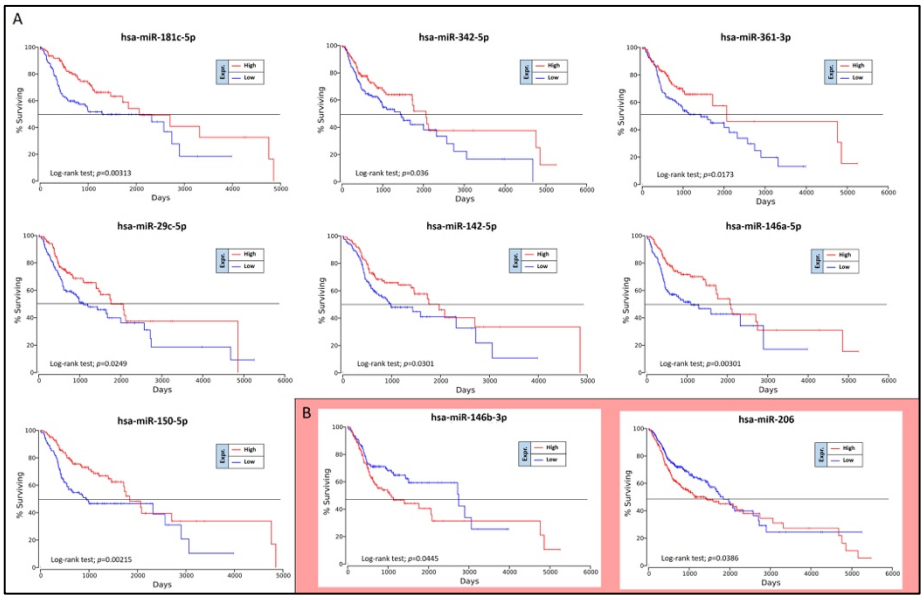


**Figure 4.5 A, B A)** Down-regulated miRs statistically correlated with patients' overall survival (OS) whose expression is in agreement with survival curves and those **B)** for which was not concordant.

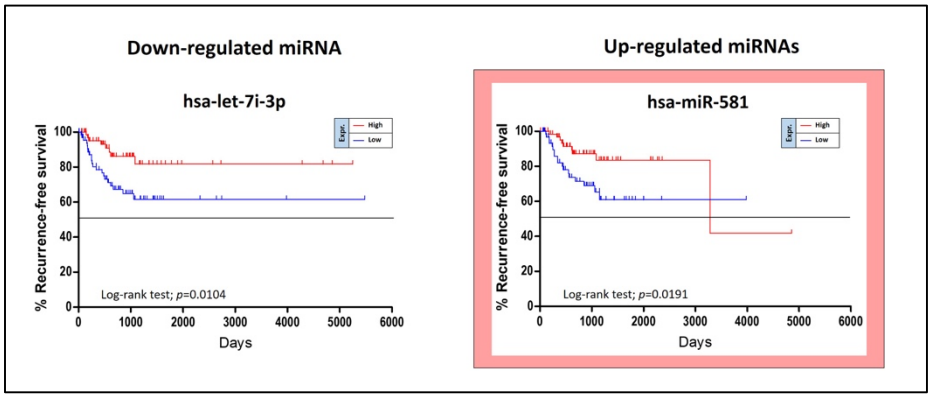


**Figure 4.6** Recurrence-free survival analysis conducted on the TCGA HNSC data.

### *4.3.7 Functional role of the 11-selected prognostic miRs: Pathway and GO Enrichment Analyses*

The same analyses (i.e. miRCancerdb and mirDIP) conducted on the 11-selected diagnostic miRs, were also performed on the selected prognostic miRs to

select the miRs-correlated and -targeted genes. The analysis showed that 19 different genes were positively and negatively correlated with the 11 miRs (**Figure 4.7A**). Moreover, the down-regulated miR-150-5p and miR-206 resulted those with the highest positive correlation levels. Interestingly, these two miRs were also those that exhibited the major level of down-regulation among the 36 differentially expressed miRs (**Table 4.6**). Furthermore, the miR-181c-5p and miR-146a-5p were the miRs more negatively connected with the identified genes.

As for the genes, the *CARD8* and *RASGAP3* genes exhibited the highest level of positive correlation with the selected miRs, while the *WDFY2, MAPK6, ESRP1,* and *PVRL1* were all negatively correlated with the 11 miRs, presenting comparable correlation levels. mirDIP analysis conducted on the 19 genes and the 11 miRs revealed no interactions for the *PVRL1* gene.

Overall, miRs exhibited a medium interaction with the selected genes. Nevertheless, the down-regulated miR-29c-5p and let-7i-3p presented reduced interaction levels with most 19 genes, while the miR-181c-5p showed the major interaction level. In addition, the most targeted gene was *CARD8* while the less was *UCP2* (**Figure 4.7B**).

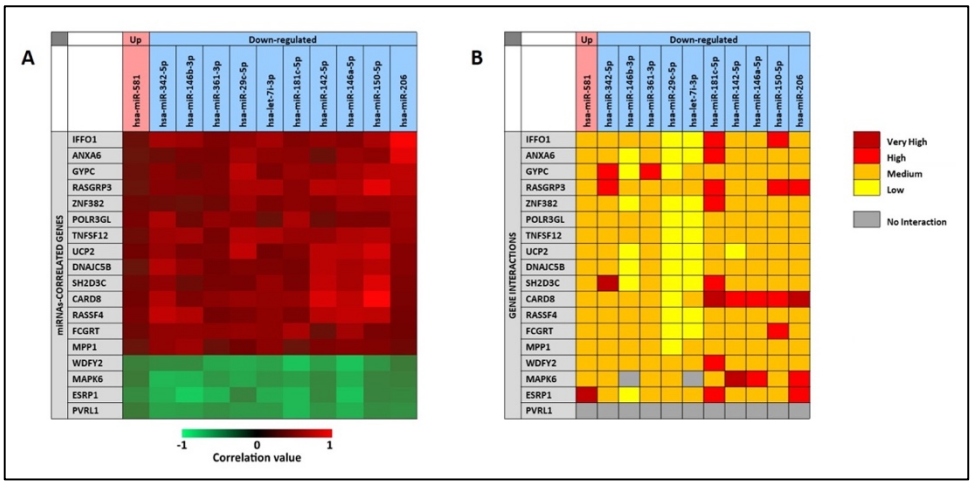


**Figure 4.7 A, B A)** miRCancerdb analysis of genes positively and negatively correlated with the 11 miRs, **B)** mirDIP analysis of interaction levels between the miRs and the altered genes.

Afterwards, the DIANA-mirPath analysis of the 11 prognostic miRs proved that the miR-581 was not able to regulate pathways and targeted genes. Conversely, the remaining 10 miRs modulated 44 different pathways and more than 1300 genes. Of note, 21 of the 44 pathways were involved in the cancer process revealing that the selected miRs regulated 292 univocal genes (**Table 4.7**).

**Table 4.7** Tumor pathways controlled by the 11-selected miRs correlated with patients' prognosis

| N. | KEGG pathway | p-value* | genes | miRs |
|---|---|---|---|---|
| **1** | PI3K-Akt signaling pathway (hsa04151) | 8.22E-03 | 82 | 10 |
| **2** | Cell cycle (hsa04110) | 1.91E-05 | 45 | 10 |
| **3** | Proteoglycans in cancer (hsa05205) | 6.81E-05 | 48 | 9 |
| **4** | Transcriptional misregulation in cancer (hsa05202) | 2.00E-02 | 46 | 9 |
| **5** | FoxO signaling pathway (hsa04068) | 2.66E-03 | 43 | 9 |
| **6** | Hippo signaling pathway (hsa04390) | 9.14E-04 | 39 | 9 |
| **7** | Melanoma (hsa05218) | 7.56E-03 | 22 | 9 |
| **8** | Viral carcinogenesis (hsa05203) | 1.08E-06 | 62 | 8 |
| **9** | Prostate cancer (hsa05215) | 6.33E-03 | 29 | 8 |
| **10** | Small cell lung cancer (hsa05222) | 2.63E-03 | 29 | 8 |
| **11** | Renal cell carcinoma (hsa05211) | 6.92E-06 | 27 | 8 |
| **12** | Chronic myeloid leukemia (hsa05220) | 7.67E-04 | 26 | 8 |
| **13** | Glioma (hsa05214) | 1.74E-04 | 23 | 8 |
| **14** | TGF-beta signaling pathway (hsa04350) | 7.03E-04 | 23 | 8 |
| **15** | Pancreatic cancer (hsa05212) | 3.18E-02 | 21 | 8 |
| **16** | Non-small cell lung cancer (hsa05223) | 8.22E-03 | 18 | 8 |
| **17** | p53 signaling pathway (hsa04115) | 2.00E-03 | 26 | 7 |
| **18** | Central carbon metabolism in cancer (hsa05230) | 1.96E-05 | 24 | 7 |
| **19** | Colorectal cancer (hsa05210) | 4.50E-02 | 19 | 6 |
| **20** | Acute myeloid leukemia (hsa05221) | 3.52E-02 | 17 | 6 |
| **21** | Endometrial cancer (hsa05213) | 4.46E-02 | 16 | 6 |

** p-values were calculated by the DIANA-mirPath, automatically employing the Fisher's Exact Test*

All the selected miRs were implicated in the control of the "PI3K-Akt signaling pathway (hsa04151)" and the "Cell cycle (hsa04110)", both linked with neoplastic transformation when de-regulated (**Table 4.7**). Interestingly, "Cell cycle (hsa04110)" pathways were also strongly modulated by the 11 cancer-associated miRs (**Table 4.5**).

Regarding the genes involved, they were all correlated with carcinogenesis including *CCND1*, *MAPK1*, *MAP2K1*, *PIK3CBPIK3R3*, *AKT2, AKT3*, *CDK4,* and *CDK6*.

Finally, the genes found through miRCancerdb and DIANA-mirPath were analyzed with GO PANTHER and STRING. Specifically, the 19 genes of miRCancerdb analysis were subject only to the GO PANTHER analysis because a greater number of genes is required for STRING analysis. For the "biological process" category, the 23.3% and 20.0% of genes were implicated in the cellular processes and in biological regulation, respectively (**Figure 4.8A**). As concerns for the "molecular function" category, the 43.5% and 17.4% of genes participated in binding and molecular regulatory functions, respectively (**Figure 4.8B**). Regarding "cellular component" category, the 19 genes encoded components of the cell, organelles, and cell junctions (37.5%, 25.0% and 18.8%, respectively) (**Figure 4.8C**).

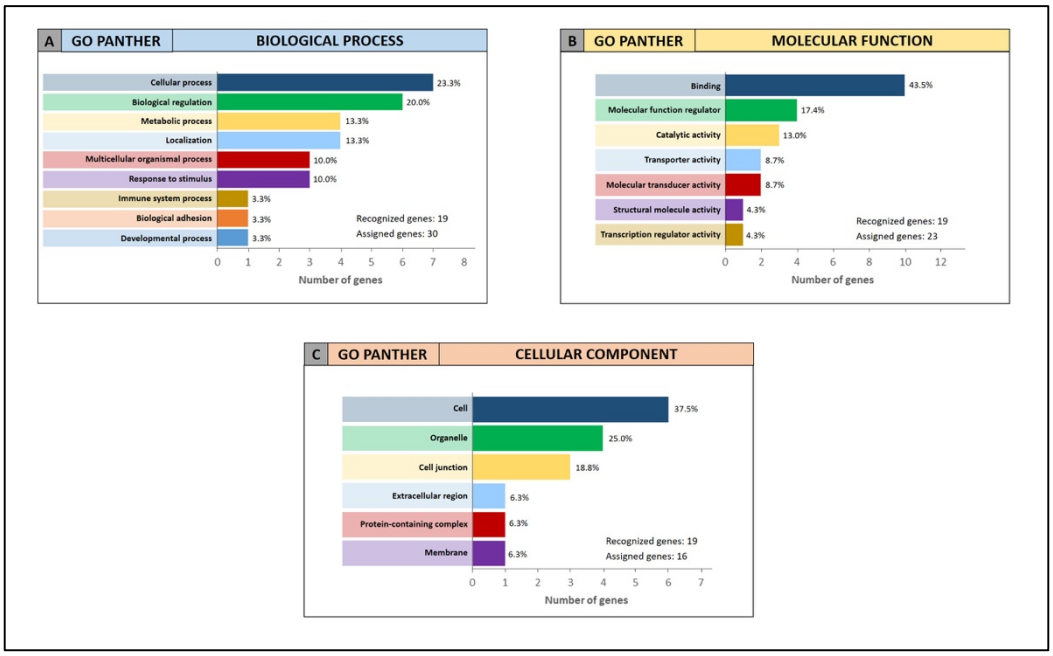


**Figure 4.8 A-C** Gene Ontology enrichment of the 19 genes selected by miRCancerdb **A)** GO PANTHER analysis of the "biological process", **B)** "molecular function", and **C)** "cellular component" category.

The same enrichment analysis was conducted on the 292 genes retrieved by DIANA-mirPath applying both GO PANTHER and STRING analyses. Regarding the "biological process" class, the majority of genes were implicated in the cellular processes and in biological regulation, as previously observed for genes identified by miRCancerdb (**Figure 9 A, D**). Also for "molecular function" and "cellular component" categories, both analyses showed results overlapping to those of genes selected with miRCancerdb (**Figure 9 B, E** and **C, F**).

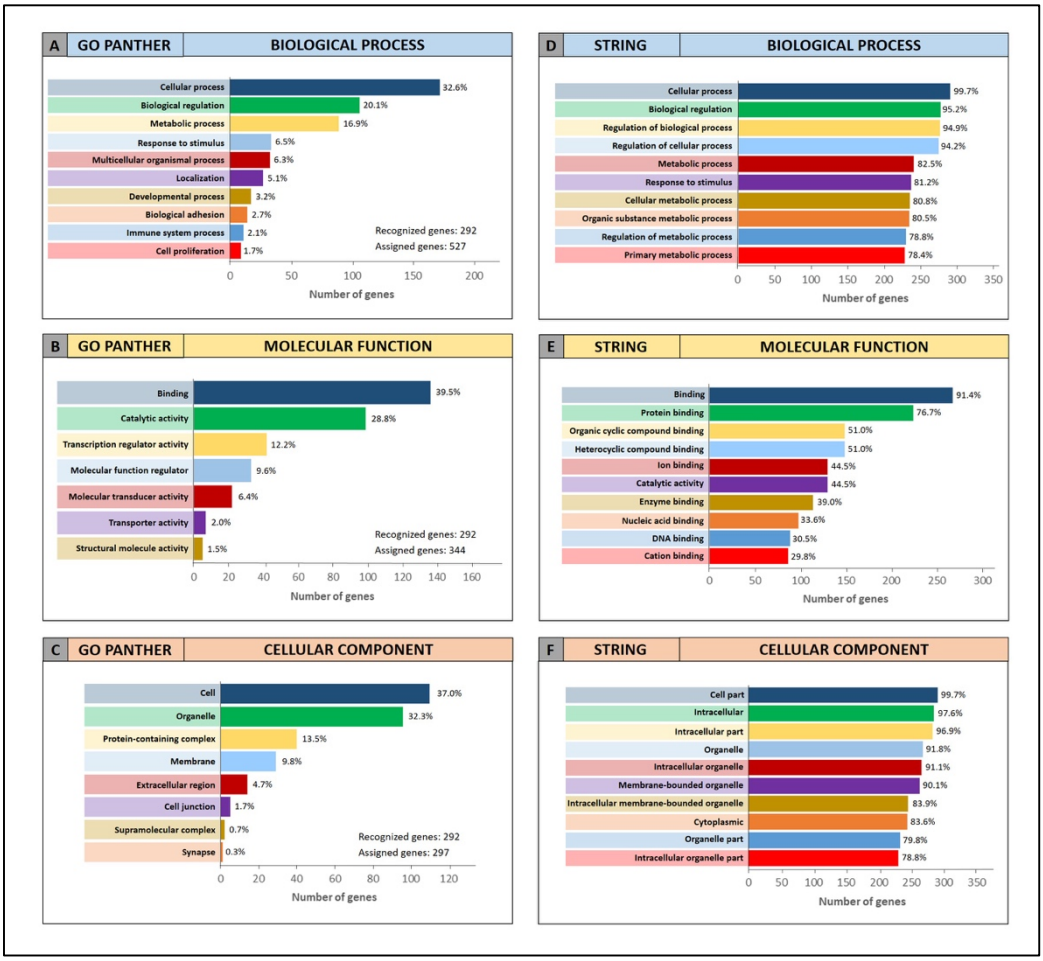


**Figure 4.9 A-F** Gene Ontology enrichment of the 292 genes selected with DIANA-mirPath. **A)** GO PANTHER and **D)** STRING analyses referred to the "biological process" category; **B)** GO PANTHER and **E)** STRING analyses related to the "molecular function" category; **C)** GO PANTHER and **F)** STRING analyses concerning the "cellular component" category.

## 4.4 Discussion

Although numerous bioinformatics data are currently available in understanding of tumors pathophysiology, the lack of a standardized approach to integrate and analyze these data produced contrasting and inconclusive results (ENCODE Project Consortium 2012, Cancer Genome Atlas Research Network *et al.* 2013, Cheng *et al.* 2015). In this context, the development of new software for the computational analysis of the so-called "big data" (Gill *et al.* 2016, Gauthier *et al.* 2018) promoted a unified and accurate bioinformatics approach to detect potential diagnostic and prognostic biomarkers of tumors, including oral cancer (Shukla 2017).

Nevertheless, the data collected in the different matrices are often provided in a different format generating difficulties in their integration and interpretation (Giacomelli & Covani 2010). Moreover, to date, no definitive diagnostic and prognostic biomarkers have been recognized for oral cancer (Santosh *et al.* 2016). Thus, the aim of the present study was to detect new specific diagnostic and prognostic biomarkers for oral cancer by applying different computational approaches to analyze and match the different miRs profiling datasets.

The two principal worldwide genomics databases (i.e. TCGA and GEO DataSets) were included to investigate the differential miR expression profiling in tumor compared to normal samples and consequently identify new potential diagnostic miRs. Moreover, the analysis on the TCGA HNSC "miRNA mature strand expression RNAseq by Illumina Hiseq" dataset permitted the detection of miRs with prognostic value. Thus, from the combination of miRs selected in both datasets, we were able to acquire potential biomarkers with a diagnostic and prognostic function.

Regarding the miRs obtained comparing cancer samples with normal, 11 de-regulated miRs have been identified, of which the up-regulated miR-196a-5p and miR-196b-5p and the down-regulated miR-99a-5p, miR-133a-3p, miR-1-3p, and miR-375-3p were the most altered. De-regulated expression of miRs has been associated with the pathogenesis of many cancers. In particular, the de-regulation of miR-196 family (miR-196a-5p and miR-196b-5p) has been correlated with carcinogenesis (Sutliff *et al.* 2019). Additionally, the miR-196 family and other miRs, including miR-375 and miR-133a-3p, have been suggested as diagnostic markers in head and neck cancers, confirming the results reported in the present search (Gissi *et al.* 2018, He *et al.* 2018, Mazumder *et al.* 2019).

Interestingly, through the differential analysis conducted on tumors at different stages, the down-regulation of these three miRs, and of miR-133a-3p seems to be linked to a more aggressive tumor phenotype (Srivastava *et al.* 2014, Li *et al.* 2015, Mizuno *et al.* 2017, Falzone *et al.* 2019). Moreover, all the miRs connected with patients' OS and RFS resulted down-regulated in high-grade tumors compared to low-grade. More specifically, the miR-150-5p, miR-181c-5p, and miR-146a-5p were the most significant miRs associated with a worse prognosis. Regarding the RFS, only the miR-let-7i-3p was a reliable indicator of disease recurrence. These data are in agreement with previous reports stating that the miR-7i (both 3p and 5p strands) negatively affects the prognosis when down-regulated (Du *et al.* 2017).

In the second part of this bioinformatics analysis, the functional role and gene association of the selected miRs was investigated. In accordance with previous reports, the DIANA-mirPath analysis revealed that the selected miRs were strongly correlated with numerous pivotal oncogenic pathways, including

*mTOR*, *p53* and *TGF*-pathways, whose implication in the oral carcinogenesis has been extensively reported (Ji *et al.* 2015, Vander Broek *et al.* 2015, Lakshminarayana *et al.* 2018). Furthermore, the selected miRs also control genes commonly down-regulated or over-expressed in oral cancer. Of note, some of these genes such as *AKT*, *BRAF*, *PIK3CA*, *NRAS*, *GSK3*, *CNND1* are implicated not only in oral carcinogenesis but in the development of different types of cancer (Vogelstein *et al*. 2013, McCubrey *et al.* 2017, Salemi *et al.* 2018). The gene ontology enrichment analyses further demonstrated that the identified genes are responsible for control and progression of biological process related to the cell proliferation, protein binding, catalytic activities, and metabolic processes. Similar results were provided by the prediction and GO enrichment analyses conducted on the 11 prognostic miRs confirming that all the miRs with a significant diagnostic and/or prognostic function can modulate cancer progression by targeting genes implicated in carcinogenic degeneration.

The advancement of both bioinformatics and molecular technologies will allow the detection of small variations in the expression levels of miRs reinforcing the function of miRs as reliable predictor of cancer (Casamassimi *et al.* 2017, Hasin *et al.* 2017, Battaglia *et al.* 2019). The computational approach applied in the present studies permitted to identify potential diagnostic and/or prognostic biomarkers through the integrated analysis of a variety of bioinformatics datasets. This multiple approach corroborates the results obtained due to the comparison and matching of different datasets. In addition, the validity of the present results is further confirmed by the findings reported by other researchers in single experimental studies.

Nevertheless, some limitations have to be underlined. First, further analyses on larger number of samples is auspicious to confirm the expression levels of these potential miRs biomarkers. Second, bioinformatics analysis should be the first step for further investigations *in vitro* and *in vivo* to validate the predictive role of selected miRs.

## 4.5 Conclusions

Within the limitations of the present study, the integrated analysis of different miR expression datasets by using various innovative bioinformatics tools allowed to identify a set of miRs all able to modulate genes and thus molecular pathways involved in cancer development and progression. More specifically, the integrated analysis allowed to identify:

1) a set of 11 de-regulated miRs with a potential diagnostic significance (i.e. hsa-miR-196a-5p, hsa-miR-196b-5p, hsa-miR-503-5p, hsa-miR-18a-5p, hsa-miR-379-5p, hsa-miR-195-5p, hsa-miR-411-5p, hsa-miR-99a-5p, hsa-miR-133a-3p, hsa-miR- 1-3p and hsa-miR-375-3p);
2) a set of 7 de-regulated miRs associated with overall survival (i.e. miR-181c-5p, miR-342-5p, miR-361-3p, miR-29c-5p, miR-142-5p, miR-146a-5p, miR-150-5p) and 1 miR to disease recurrence (i.e. mir-7i-3p);
3) a set of 4 de-regulated miRs with both potential diagnostic and prognostic role (i.e. miR-139-3p, miR-142-5p and miR-29c-3p, and miR-133a-3p.

The above-mentioned miRs could be indicators for an early detection and/or progression of cancerous oral lesions, after that their use be validated *in vitro* and *in vivo* investigations.

## 4.6 Supplementary materials

**Table S4.1** Full list of 514 de-regulated TCGA HNSC miRs correlated with cancer.

| miR ID | miR name | FC Cancer vs Normal | p-value* |
|---|---|---|---|
| **MIMAT0000226** | **hsa-miR-196a-5p** | 12.145 | 3.12E-19 |
| **MIMAT0001080** | **hsa-miR-196b-5p** | 11.639 | 5.43E-20 |
| **MIMAT0000267** | **hsa-miR-210-3p** | 9.733 | 1.18E-09 |
| **MIMAT0000089** | **hsa-miR-31-5p** | 7.684 | 8.42E-12 |
| **MIMAT0004784** | **hsa-miR-455-3p** | 7.165 | 9.21E-18 |
| **MIMAT0005923** | **hsa-miR-1269a** | 5.899 | 1.99E-11 |
| **MIMAT0000102** | **hsa-miR-105-5p** | 5.510 | 9.64E-13 |
| **MIMAT0004504** | **hsa-miR-31-3p** | 5.298 | 1.59E-09 |
| **MIMAT0003882** | **hsa-miR-767-5p** | 5.294 | 5.40E-13 |
| **MIMAT0000281** | **hsa-miR-224-5p** | 4.789 | 5.39E-11 |
| **MIMAT0002874** | **hsa-miR-503-5p** | 4.044 | 3.86E-19 |
| **MIMAT0002819** | **hsa-miR-193b-3p** | 3.407 | 8.17E-15 |
| **MIMAT0005951** | **hsa-miR-1307-3p** | 3.395 | 1.14E-11 |
| **MIMAT0000076** | **hsa-miR-21-5p** | 3.209 | 3.05E-10 |
| **MIMAT0000266** | **hsa-miR-205-5p** | 3.040 | 1.64E-05 |
| **MIMAT0016895** | **hsa-miR-2355-5p** | 3.023 | 6.22E-14 |
| **MIMAT0004987** | **hsa-miR-944** | 3.020 | 7.56E-07 |
| **MIMAT0005797** | **hsa-miR-1301-3p** | 2.902 | 6.39E-17 |
| **MIMAT0000761** | **hsa-miR-324-5p** | 2.878 | 7.41E-12 |
| **MIMAT0000758** | **hsa-miR-135b-5p** | 2.859 | 4.08E-08 |
| **MIMAT0001341** | **hsa-miR-424-5p** | 2.856 | 4.57E-13 |
| **MIMAT0000072** | **hsa-miR-18a-5p** | 2.829 | 8.10E-10 |
| **MIMAT0001545** | **hsa-miR-450a-5p** | 2.828 | 1.20E-15 |
| **MIMAT0000688** | **hsa-miR-301a-3p** | 2.807 | 5.32E-13 |
| **MIMAT0003150** | **hsa-miR-455-5p** | 2.799 | 3.50E-12 |
| **MIMAT0000093** | hsa-miR-93-5p | 2.792 | 3.47E-08 |
| **MIMAT0004494** | hsa-miR-21-3p | 2.787 | 8.08E-07 |
| **MIMAT0016888** | hsa-miR-4326 | 2.781 | 4.82E-12 |
| **MIMAT0004980** | hsa-miR-937-3p | 2.757 | 3.29E-24 |
| **MIMAT0019716** | hsa-miR-4652-5p | 2.722 | 8.81E-11 |
| **MIMAT0004680** | hsa-miR-130b-5p | 2.701 | 1.81E-14 |
| **MIMAT0000261** | hsa-miR-183-5p | 2.685 | 8.21E-15 |
| **MIMAT0000070** | hsa-miR-17-5p | 2.662 | 8.05E-08 |
| **MIMAT0017950** | hsa-miR-2355-3p | 2.646 | 1.04E-11 |
| **MIMAT0004509** | hsa-miR-93-3p | 2.595 | 4.38E-17 |
| **MIMAT0003880** | hsa-miR-671-5p | 2.582 | 8.52E-16 |
| **MIMAT0004926** | hsa-miR-708-5p | 2.578 | 1.04E-06 |
| **MIMAT0005883** | hsa-miR-1293 | 2.556 | 3.93E-08 |
| **MIMAT0022696** | hsa-miR-301a-5p | 2.551 | 9.18E-18 |
| **MIMAT0019814** | hsa-miR-203b-3p | 2.487 | 1.78E-04 |
| **MIMAT0004496** | hsa-miR-23a-5p | 2.441 | 3.75E-10 |
| **MIMAT0004749** | hsa-miR-424-3p | 2.440 | 1.72E-20 |

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|---|---|---|---|
| **MIMAT0004497** | hsa-miR-24-2-5p | 2.421 | 4.61E-07 |
| **MIMAT0003283** | hsa-miR-615-3p | 2.396 | 6.03E-08 |
| **MIMAT0000691** | hsa-miR-130b-3p | 2.396 | 7.34E-21 |
| **MIMAT0004697** | hsa-miR-151a-5p | 2.396 | 1.96E-15 |
| **MIMAT0004909** | hsa-miR-450b-5p | 2.382 | 2.02E-11 |
| **MIMAT0004927** | hsa-miR-708-3p | 2.352 | 1.88E-06 |
| **MIMAT0000262** | hsa-miR-187-3p | 2.351 | 6.80E-07 |
| **MIMAT0003885** | hsa-miR-454-3p | 2.346 | 1.21E-10 |
| **MIMAT0000073** | hsa-miR-19a-3p | 2.332 | 1.76E-06 |
| **MIMAT0000279** | hsa-miR-222-3p | 2.299 | 2.84E-11 |
| **MIMAT0019880** | hsa-miR-4746-5p | 2.279 | 8.71E-17 |
| **MIMAT0000095** | hsa-miR-96-5p | 2.269 | 3.96E-12 |
| **MIMAT0001340** | hsa-miR-423-3p | 2.252 | 2.08E-09 |
| **MIMAT0009199** | hsa-miR-365a-5p | 2.251 | 1.87E-28 |
| **MIMAT0004514** | hsa-miR-29b-1-5p | 2.208 | 1.01E-08 |
| **MIMAT0004767** | hsa-miR-193b-5p | 2.201 | 2.61E-14 |
| **MIMAT0004949** | hsa-miR-877-5p | 2.190 | 7.24E-28 |
| **MIMAT0009197** | hsa-miR-205-3p | 2.148 | 6.33E-07 |
| **MIMAT0004946** | hsa-miR-744-3p | 2.144 | 6.64E-09 |
| **MIMAT0000075** | hsa-miR-20a-5p | 2.125 | 4.90E-05 |
| **MIMAT0004672** | hsa-miR-106b-3p | 2.122 | 1.26E-13 |
| **MIMAT0000259** | hsa-miR-182-5p | 2.114 | 1.15E-09 |
| **MIMAT0003298** | hsa-miR-629-3p | 2.109 | 2.52E-13 |
| **MIMAT0001636** | hsa-miR-452-3p | 2.093 | 6.90E-10 |
| **MIMAT0000252** | hsa-miR-7-5p | 2.065 | 2.35E-13 |
| **MIMAT0004586** | hsa-miR-15b-3p | 2.060 | 4.01E-11 |
| **MIMAT0002891** | hsa-miR-18a-3p | 2.050 | 4.52E-17 |
| **MIMAT0000257** | hsa-miR-181b-5p | 2.047 | 7.26E-14 |
| **MIMAT0000764** | hsa-miR-339-5p | 2.034 | 1.83E-05 |
| **MIMAT0022726** | hsa-miR-1306-5p | 2.024 | 1.97E-12 |
| **MIMAT0004553** | hsa-miR-7-1-3p | 2.022 | 6.46E-10 |
| **MIMAT0000434** | hsa-miR-142-3p | 2.011 | 2.27E-04 |
| **MIMAT0004493** | hsa-miR-20a-3p | 2.010 | 4.61E-07 |
| **MIMAT0004657** | hsa-miR-200c-5p | 1.993 | 3.86E-05 |
| **MIMAT0002174** | hsa-miR-484 | 1.984 | 2.29E-08 |
| **MIMAT0022692** | hsa-miR-181b-3p | 1.974 | 2.20E-07 |
| **MIMAT0004507** | hsa-miR-92a-1-5p | 1.961 | 5.47E-08 |
| **MIMAT0004693** | hsa-miR-330-5p | 1.961 | 2.54E-09 |
| **MIMAT0003218** | hsa-miR-92b-3p | 1.959 | 4.29E-05 |
| **MIMAT0000092** | hsa-miR-92a-3p | 1.958 | 2.34E-08 |
| **MIMAT0004518** | hsa-miR-16-2-3p | 1.941 | 7.90E-11 |
| **MIMAT0004776** | hsa-miR-505-5p | 1.939 | 7.85E-08 |
| **MIMAT0005825** | hsa-miR-1180-3p | 1.930 | 7.89E-08 |
| **MIMAT0003257** | hsa-miR-550a-3p | 1.905 | 2.28E-17 |
| **MIMAT0005895** | hsa-miR-548f-3p | 1.904 | 7.25E-26 |
| **MIMAT0018101** | hsa-miR-3677-3p | 1.896 | 4.77E-10 |
| **MIMAT0003393** | hsa-miR-425-5p | 1.892 | 5.30E-05 |
| **MIMAT0000760** | hsa-miR-331-3p | 1.886 | 1.75E-10 |

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|---|---|---|---|
| **MIMAT0000441** | hsa-miR-9-5p | 1.885 | 6.13E-04 |
| **MIMAT0022925** | hsa-miR-503-3p | 1.879 | 1.34E-22 |
| **MIMAT0000091** | hsa-miR-33a-5p | 1.867 | 2.45E-03 |
| **MIMAT0000753** | hsa-miR-342-3p | 1.857 | 7.44E-05 |
| **MIMAT0003888** | hsa-miR-766-3p | 1.856 | 2.52E-14 |
| **MIMAT0017992** | hsa-miR-3614-5p | 1.856 | 1.12E-07 |
| **MIMAT0000227** | hsa-miR-197-3p | 1.853 | 4.66E-09 |
| **MIMAT0002876** | hsa-miR-505-3p | 1.846 | 5.66E-10 |
| **MIMAT0005792** | hsa-miR-320b | 1.832 | 8.47E-09 |
| **MIMAT0003241** | hsa-miR-576-5p | 1.823 | 3.33E-09 |
| **MIMAT0000762** | hsa-miR-324-3p | 1.822 | 2.23E-10 |
| **MIMAT0002809** | hsa-miR-146b-5p | 1.820 | 1.11E-03 |
| **MIMAT0004678** | hsa-miR-99b-3p | 1.801 | 6.51E-17 |
| **MIMAT0001620** | hsa-miR-200a-5p | 1.796 | 3.22E-04 |
| **MIMAT0019208** | hsa-miR-3074-5p | 1.779 | 2.81E-10 |
| **MIMAT0003249** | hsa-miR-584-5p | 1.777 | 1.37E-07 |
| **MIMAT0004983** | hsa-miR-940 | 1.775 | 1.36E-07 |
| **MIMAT0004800** | hsa-miR-550a-5p | 1.773 | 7.08E-11 |
| **MIMAT0018349** | hsa-miR-3934-5p | 1.769 | 3.82E-17 |
| **MIMAT0022720** | hsa-miR-1304-3p | 1.766 | 1.97E-10 |
| **MIMAT0004799** | hsa-miR-589-5p | 1.763 | 3.85E-19 |
| **MIMAT0003256** | hsa-miR-589-3p | 1.761 | 1.82E-11 |
| **MIMAT0000256** | hsa-miR-181a-5p | 1.757 | 2.35E-09 |
| **MIMAT0003260** | hsa-miR-592 | 1.744 | 4.62E-11 |
| **MIMAT0007884** | hsa-miR-1910-5p | 1.735 | 3.26E-07 |
| **MIMAT0023712** | hsa-miR-6087 | 1.731 | 6.86E-04 |
| **MIMAT0004484** | hsa-let-7d-3p | 1.713 | 2.22E-08 |
| **MIMAT0004774** | hsa-miR-501-3p | 1.710 | 1.20E-05 |
| **MIMAT0015050** | hsa-miR-323b-3p | 1.698 | 6.30E-08 |
| **MIMAT0003389** | hsa-miR-542-3p | 1.688 | 1.63E-05 |
| **MIMAT0000772** | hsa-miR-345-5p | 1.676 | 4.23E-06 |
| **MIMAT0009196** | hsa-miR-103a-2-5p | 1.676 | 4.88E-07 |
| **MIMAT0000417** | hsa-miR-15b-5p | 1.675 | 1.58E-08 |
| **MIMAT0005893** | hsa-miR-1305 | 1.674 | 6.66E-14 |
| **MIMAT0005452** | hsa-miR-519a-5p | 1.669 | 1.50E-09 |
| **MIMAT0000510** | hsa-miR-320a-3p | 1.669 | 2.26E-06 |
| **MIMAT0000068** | hsa-miR-15a-5p | 1.668 | 1.70E-04 |
| **MIMAT0004780** | hsa-miR-532-3p | 1.667 | 1.03E-06 |
| **MIMAT0000425** | hsa-miR-130a-3p | 1.657 | 1.47E-07 |
| **MIMAT0030020** | hsa-miR-7705 | 1.656 | 3.74E-16 |
| **MIMAT0022975** | hsa-miR-3934-3p | 1.653 | 2.33E-10 |
| **MIMAT0025450** | hsa-miR-6499-5p | 1.648 | 2.61E-05 |
| **MIMAT0025451** | hsa-miR-6499-3p | 1.646 | 6.77E-04 |
| **MIMAT0018083** | hsa-miR-3662 | 1.644 | 2.54E-07 |
| **MIMAT0000432** | hsa-miR-141-3p | 1.635 | 3.49E-03 |
| **MIMAT0004700** | hsa-miR-331-5p | 1.631 | 2.09E-12 |
| **MIMAT0004489** | hsa-miR-16-1-3p | 1.621 | 3.31E-05 |
| **MIMAT0000455** | hsa-miR-185-5p | 1.609 | 5.33E-07 |

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|---|---|---|---|
| **MIMAT0003339** | hsa-miR-421 | 1.606 | 1.97E-11 |
| **MIMAT0004495** | hsa-miR-22-5p | 1.596 | 5.59E-04 |
| **MIMAT0004694** | hsa-miR-342-5p | 1.596 | 5.58E-07 |
| **MIMAT0017994** | hsa-miR-3615 | 1.585 | 4.64E-07 |
| **MIMAT0003258** | hsa-miR-590-5p | 1.559 | 1.25E-05 |
| **MIMAT0026479** | hsa-miR-152-5p | 1.559 | 6.10E-11 |
| **MIMAT0030017** | hsa-miR-7702 | 1.557 | 7.07E-09 |
| **MIMAT0018197** | hsa-miR-3922-3p | 1.554 | 5.93E-12 |
| **MIMAT0004593** | hsa-miR-130a-5p | 1.542 | 8.02E-16 |
| **MIMAT0030021** | hsa-miR-7706 | 1.541 | 5.66E-08 |
| **MIMAT0000617** | hsa-miR-200c-3p | 1.540 | 5.72E-03 |
| **MIMAT0004611** | hsa-miR-185-3p | 1.536 | 1.81E-07 |
| **MIMAT0009451** | hsa-miR-1976 | 1.533 | 2.49E-07 |
| **MIMAT0004770** | hsa-miR-516a-5p | 1.532 | 3.73E-06 |
| **MIMAT0004698** | hsa-miR-135b-3p | 1.529 | 1.39E-06 |
| **MIMAT0004491** | hsa-miR-19b-1-5p | 1.519 | 6.36E-05 |
| **MIMAT0004486** | hsa-let-7f-1-3p | 1.518 | 2.70E-09 |
| **MIMAT0004558** | hsa-miR-181a-2-3p | 1.511 | 2.40E-07 |
| **MIMAT0000270** | hsa-miR-181a-3p | 1.508 | 2.95E-05 |
| **MIMAT0000081** | hsa-miR-25-3p | 1.503 | 1.33E-06 |
| **MIMAT0002872** | hsa-miR-501-5p | 1.497 | 2.55E-05 |
| **MIMAT0004588** | hsa-miR-27b-5p | 1.497 | 5.32E-05 |
| **MIMAT0001635** | hsa-miR-452-5p | 1.495 | 1.13E-03 |
| **MIMAT0004985** | hsa-miR-942-5p | 1.494 | 4.66E-06 |
| **MIMAT0003284** | hsa-miR-616-5p | 1.487 | 1.74E-07 |
| **MIMAT0000440** | hsa-miR-191-5p | 1.485 | 4.74E-06 |
| **MIMAT0004699** | hsa-miR-148b-5p | 1.483 | 3.81E-09 |
| **MIMAT0018115** | hsa-miR-3687 | 1.483 | 2.78E-08 |
| **MIMAT0005905** | hsa-miR-1254 | 1.479 | 6.76E-10 |
| **MIMAT0003312** | hsa-miR-642a-5p | 1.468 | 1.19E-05 |
| **MIMAT0019746** | hsa-miR-4668-3p | 1.466 | 2.33E-06 |
| **MIMAT0004560** | hsa-miR-183-3p | 1.462 | 1.20E-10 |
| **MIMAT0022727** | hsa-miR-1307-5p | 1.461 | 4.07E-03 |
| **MIMAT0002173** | hsa-miR-483-3p | 1.459 | 2.99E-04 |
| **MIMAT0025851** | hsa-miR-6720-3p | 1.459 | 1.94E-04 |
| **MIMAT0019738** | hsa-miR-4664-3p | 1.455 | 1.16E-07 |
| **MIMAT0004584** | hsa-let-7g-3p | 1.454 | 1.81E-03 |
| **MIMAT0005950** | hsa-miR-1306-3p | 1.453 | 7.51E-10 |
| **MIMAT0018068** | hsa-miR-3648 | 1.449 | 8.47E-03 |
| **MIMAT0000101** | hsa-miR-103a-3p | 1.445 | 3.95E-05 |
| **MIMAT0000429** | hsa-miR-137-3p | 1.443 | 8.24E-05 |
| **MIMAT0000278** | hsa-miR-221-3p | 1.443 | 3.20E-04 |
| **MIMAT0000755** | hsa-miR-323a-3p | 1.439 | 5.31E-05 |
| **MIMAT0004958** | hsa-miR-301b-3p | 1.439 | 1.72E-09 |
| **MIMAT0004982** | hsa-miR-939-5p | 1.437 | 1.20E-13 |
| **MIMAT0004810** | hsa-miR-629-5p | 1.436 | 7.65E-05 |
| **MIMAT0004773** | hsa-miR-500a-5p | 1.435 | 6.40E-04 |
| **MIMAT0016925** | hsa-miR-500b-5p | 1.435 | 6.57E-04 |

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| **MIMAT0022833** | hsa-miR-365b-5p | 1.429 | 2.09E-10 |
| **MIMAT0003242** | hsa-miR-577 | 1.429 | 6.85E-03 |
| **MIMAT0004599** | hsa-miR-143-5p | 1.425 | 4.84E-03 |
| **MIMAT0002823** | hsa-miR-512-3p | 1.425 | 2.35E-06 |
| **MIMAT0005933** | hsa-miR-1277-3p | 1.423 | 1.65E-08 |
| **MIMAT0027682** | hsa-miR-6891-5p | 1.423 | 2.84E-07 |
| **MIMAT0026477** | hsa-miR-128-1-5p | 1.422 | 2.48E-05 |
| **MIMAT0000689** | hsa-miR-99b-5p | 1.421 | 6.70E-07 |
| **MIMAT0022834** | hsa-miR-365b-3p | 1.413 | 4.07E-03 |
| **MIMAT0000710** | hsa-miR-365a-3p | 1.413 | 4.10E-03 |
| **MIMAT0004945** | hsa-miR-744-5p | 1.412 | 1.57E-05 |
| **MIMAT0026475** | hsa-miR-210-5p | 1.411 | 1.60E-06 |
| **MIMAT0019761** | hsa-miR-4677-3p | 1.400 | 5.82E-08 |
| **MIMAT0018968** | hsa-miR-4449 | 1.398 | 1.23E-09 |
| **MIMAT0018205** | hsa-miR-3928-3p | 1.398 | 6.43E-06 |
| **MIMAT0003887** | hsa-miR-769-3p | 1.397 | 3.01E-08 |
| **MIMAT0018071** | hsa-miR-3651 | 1.397 | 2.42E-03 |
| **MIMAT0018993** | hsa-miR-4466 | 1.395 | 6.02E-28 |
| **MIMAT0019927** | hsa-miR-4772-3p | 1.391 | 3.39E-03 |
| **MIMAT0004748** | hsa-miR-423-5p | 1.388 | 9.00E-05 |
| **MIMAT0027608** | hsa-miR-6854-5p | 1.384 | 3.08E-03 |
| **MIMAT0009198** | hsa-miR-224-3p | 1.384 | 3.24E-05 |
| **MIMAT0018110** | hsa-miR-3682-3p | 1.384 | 8.34E-08 |
| **MIMAT0005901** | hsa-miR-1249-3p | 1.384 | 3.96E-05 |
| **MIMAT0018356** | hsa-miR-3940-3p | 1.382 | 3.90E-08 |
| **MIMAT0002808** | hsa-miR-511-5p | 1.381 | 4.31E-04 |
| **MIMAT0000078** | hsa-miR-23a-3p | 1.375 | 8.23E-07 |
| **MIMAT0017982** | hsa-miR-3605-3p | 1.371 | 8.31E-11 |
| **MIMAT0027587** | hsa-miR-6842-3p | 1.367 | 2.59E-06 |
| **MIMAT0004658** | hsa-miR-155-3p | 1.365 | 1.72E-07 |
| **MIMAT0004567** | hsa-miR-219a-1-3p | 1.361 | 6.75E-04 |
| **MIMAT0000069** | hsa-miR-16-5p | 1.361 | 6.36E-05 |
| **MIMAT0031177** | hsa-miR-7974 | 1.356 | 4.01E-04 |
| **MIMAT0000084** | hsa-miR-27a-3p | 1.356 | 2.02E-04 |
| **MIMAT0018191** | hsa-miR-3917 | 1.353 | 7.62E-05 |
| **MIMAT0004911** | hsa-miR-874-3p | 1.352 | 3.58E-03 |
| **MIMAT0026734** | hsa-miR-942-3p | 1.351 | 2.24E-08 |
| **MIMAT0017352** | hsa-miR-2277-5p | 1.345 | 4.29E-10 |
| **MIMAT0003884** | hsa-miR-454-5p | 1.340 | 6.52E-09 |
| **MIMAT0004498** | hsa-miR-25-5p | 1.339 | 1.68E-13 |
| **MIMAT0000065** | hsa-let-7d-5p | 1.337 | 3.09E-06 |
| **MIMAT0001618** | hsa-miR-191-3p | 1.336 | 1.84E-05 |
| **MIMAT0003340** | hsa-miR-542-5p | 1.335 | 3.17E-05 |
| **MIMAT0004614** | hsa-miR-193a-5p | 1.329 | 3.49E-04 |
| **MIMAT0021021** | hsa-miR-5001-5p | 1.327 | 1.32E-09 |
| **MIMAT0019820** | hsa-miR-4713-5p | 1.325 | 3.56E-07 |
| **MIMAT0019696** | hsa-miR-4638-3p | 1.322 | 9.16E-08 |
| **MIMAT0015045** | hsa-miR-3170 | 1.318 | 3.56E-08 |

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| **MIMAT0000759** | hsa-miR-148b-3p | 1.317 | 3.71E-05 |
| **MIMAT0017990** | hsa-miR-3613-5p | 1.316 | 6.12E-03 |
| **MIMAT0019926** | hsa-miR-4772-5p | 1.316 | 1.08E-03 |
| **MIMAT0002847** | hsa-miR-518c-5p | 1.315 | 6.37E-04 |
| **MIMAT0004761** | hsa-miR-483-5p | 1.310 | 4.99E-03 |
| **MIMAT0026738** | hsa-miR-1287-3p | 1.309 | 2.67E-03 |
| **MIMAT0019229** | hsa-miR-3940-5p | 1.308 | 1.05E-03 |
| **MIMAT0005943** | hsa-miR-1292-5p | 1.306 | 5.37E-07 |
| **MIMAT0014990** | hsa-miR-3127-5p | 1.305 | 6.28E-03 |
| **MIMAT0003322** | hsa-miR-652-3p | 1.305 | 2.75E-04 |
| **MIMAT0019729** | hsa-miR-4661-5p | 1.304 | 1.98E-04 |
| **MIMAT0004503** | hsa-miR-29a-5p | 1.304 | 2.14E-03 |
| **MIMAT0019776** | hsa-miR-1343-3p | 1.303 | 8.78E-11 |
| **MIMAT0019725** | hsa-miR-4658 | 1.300 | 1.66E-03 |
| **MIMAT0010195** | hsa-let-7a-2-3p | 1.297 | 5.52E-03 |
| **MIMAT0019221** | hsa-miR-3677-5p | 1.296 | 7.78E-05 |
| **MIMAT0000456** | hsa-miR-186-5p | 1.295 | 4.07E-05 |
| **MIMAT0000680** | hsa-miR-106b-5p | 1.292 | 1.42E-03 |
| **MIMAT0019958** | hsa-miR-4788 | 1.288 | 1.69E-05 |
| **MIMAT0005796** | hsa-miR-1271-5p | 1.287 | 1.25E-03 |
| **MIMAT0022708** | hsa-miR-584-3p | 1.286 | 1.11E-06 |
| **MIMAT0000457** | hsa-miR-188-5p | 1.282 | 1.06E-03 |
| **MIMAT0003326** | hsa-miR-663a | 1.277 | 5.04E-06 |
| **MIMAT0004671** | hsa-miR-194-3p | 1.274 | 7.22E-05 |
| **MIMAT0004957** | hsa-miR-760 | 1.264 | 1.10E-06 |
| **MIMAT0018120** | hsa-miR-3691-5p | 1.263 | 1.65E-08 |
| **MIMAT0004502** | hsa-miR-28-3p | 1.262 | 2.92E-04 |
| **MIMAT0018119** | hsa-miR-3690 | 1.259 | 4.05E-03 |
| **MIMAT0002821** | hsa-miR-181d-5p | 1.258 | 2.24E-04 |
| **MIMAT0030429** | hsa-miR-7854-3p | 1.253 | 4.52E-05 |
| **MIMAT0014979** | hsa-miR-3117-3p | 1.253 | 1.10E-04 |
| **MIMAT0005882** | hsa-miR-548k | 1.251 | 1.27E-04 |
| **MIMAT0004682** | hsa-miR-361-3p | 1.248 | 1.25E-03 |
| **MIMAT0015053** | hsa-miR-3176 | 1.248 | 4.38E-18 |
| **MIMAT0022270** | hsa-miR-5579-3p | 1.247 | 1.14E-03 |
| **MIMAT0002844** | hsa-miR-518b | 1.246 | 5.61E-04 |
| **MIMAT0004679** | hsa-miR-296-3p | 1.245 | 2.15E-04 |
| **MIMAT0002830** | hsa-miR-520f-3p | 1.244 | 1.32E-03 |
| **MIMAT0021044** | hsa-miR-5010-3p | 1.241 | 1.09E-06 |
| **MIMAT0017993** | hsa-miR-3614-3p | 1.238 | 2.80E-03 |
| **MIMAT0005577** | hsa-miR-1226-3p | 1.232 | 2.27E-06 |
| **MIMAT0004819** | hsa-miR-671-3p | 1.222 | 2.99E-03 |
| **MIMAT0018107** | hsa-miR-3680-3p | 1.221 | 3.35E-06 |
| **MIMAT0005584** | hsa-miR-1229-3p | 1.215 | 1.11E-05 |
| **MIMAT0000222** | hsa-miR-192-5p | 1.215 | 2.44E-03 |
| **MIMAT0026718** | hsa-miR-874-5p | 1.215 | 1.19E-06 |
| **MIMAT0004928** | hsa-miR-147b-3p | 1.214 | 2.35E-03 |
| **MIMAT0004505** | hsa-miR-32-3p | 1.211 | 5.40E-04 |

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|---|---|---|---|
| **MIMAT0019841** | hsa-miR-4724-5p | 1.210 | 3.38E-03 |
| **MIMAT0015069** | hsa-miR-3187-3p | 1.210 | 5.54E-08 |
| **MIMAT0004805** | hsa-miR-616-3p | 1.204 | 1.09E-04 |
| **MIMAT0003287** | hsa-miR-618 | 1.202 | 4.05E-04 |
| **MIMAT0004956** | hsa-miR-374b-3p | 1.202 | 1.99E-03 |
| **MIMAT0019918** | hsa-miR-4766-3p | 1.197 | 6.48E-03 |
| **MIMAT0027357** | hsa-miR-6728-5p | 1.195 | 4.87E-03 |
| **MIMAT0019214** | hsa-miR-3173-5p | 1.194 | 2.26E-05 |
| **MIMAT0018187** | hsa-miR-3913-5p | 1.193 | 5.52E-03 |
| **MIMAT0030425** | hsa-miR-7850-5p | 1.193 | 1.47E-05 |
| **MIMAT0002858** | hsa-miR-520g-3p | 1.191 | 2.76E-03 |
| **MIMAT0019202** | hsa-miR-3129-3p | 1.191 | 8.04E-04 |
| **MIMAT0005583** | hsa-miR-1228-3p | 1.186 | 3.37E-03 |
| **MIMAT0022710** | hsa-miR-659-5p | 1.185 | 3.89E-03 |
| **MIMAT0022496** | hsa-miR-5703 | 1.184 | 1.11E-05 |
| **MIMAT0028122** | hsa-miR-7112-3p | 1.184 | 3.54E-04 |
| **MIMAT0027486** | hsa-miR-6793-5p | 1.181 | 2.06E-04 |
| **MIMAT0030419** | hsa-miR-7844-5p | 1.181 | 2.28E-06 |
| **MIMAT0005942** | hsa-miR-1288-3p | 1.180 | 2.24E-06 |
| **MIMAT0019737** | hsa-miR-4664-5p | 1.179 | 2.57E-03 |
| **MIMAT0017987** | hsa-miR-3610 | 1.179 | 8.76E-03 |
| **MIMAT0019873** | hsa-miR-4742-3p | 1.179 | 1.16E-04 |
| **MIMAT0018185** | hsa-miR-3911 | 1.178 | 9.36E-07 |
| **MIMAT0026616** | hsa-miR-579-5p | 1.178 | 7.45E-05 |
| **MIMAT0005930** | hsa-miR-1276 | 1.177 | 7.42E-06 |
| **MIMAT0022842** | hsa-miR-98-3p | 1.177 | 2.03E-03 |
| **MIMAT0003309** | hsa-miR-639 | 1.174 | 2.32E-05 |
| **MIMAT0015003** | hsa-miR-3136-5p | 1.172 | 9.82E-04 |
| **MIMAT0027032** | hsa-miR-500b-3p | 1.170 | 5.36E-03 |
| **MIMAT0005875** | hsa-miR-548j-5p | 1.169 | 7.97E-04 |
| **MIMAT0002838** | hsa-miR-525-5p | 1.166 | 5.02E-03 |
| **MIMAT0027604** | hsa-miR-6852-5p | 1.166 | 1.33E-03 |
| **MIMAT0019706** | hsa-miR-4645-3p | 1.166 | 2.56E-05 |
| **MIMAT0004607** | hsa-miR-138-1-3p | 1.164 | 3.44E-04 |
| **MIMAT0004488** | hsa-miR-15a-3p | 1.163 | 7.12E-05 |
| **MIMAT0004785** | hsa-miR-545-5p | 1.163 | 1.17E-04 |
| **MIMAT0022698** | hsa-miR-345-3p | 1.161 | 3.24E-05 |
| **MIMAT0017991** | hsa-miR-3613-3p | 1.160 | 2.31E-03 |
| **MIMAT0027454** | hsa-miR-6777-5p | 1.155 | 2.54E-03 |
| **MIMAT0019935** | hsa-miR-4777-3p | 1.153 | 2.04E-04 |
| **MIMAT0019871** | hsa-miR-4741 | 1.152 | 8.31E-07 |
| **MIMAT0027541** | hsa-miR-6820-3p | 1.152 | 7.63E-05 |
| **MIMAT0003306** | hsa-miR-636 | 1.152 | 3.68E-06 |
| **MIMAT0020925** | hsa-miR-550a-3-5p | 1.149 | 9.21E-04 |
| **MIMAT0019728** | hsa-miR-4660 | 1.149 | 7.67E-03 |
| **MIMAT0003235** | hsa-miR-570-3p | 1.146 | 1.47E-03 |
| **MIMAT0005892** | hsa-miR-1304-5p | 1.145 | 2.30E-03 |
| **MIMAT0005924** | hsa-miR-1270 | 1.145 | 3.74E-03 |

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| **MIMAT0011777** | hsa-miR-2277-3p | 1.144 | 8.21E-04 |
| **MIMAT0027497** | hsa-miR-6798-3p | 1.144 | 2.26E-05 |
| **MIMAT0022709** | hsa-miR-652-5p | 1.142 | 2.54E-03 |
| **MIMAT0005881** | hsa-miR-1291 | 1.141 | 2.78E-04 |
| **MIMAT0018360** | hsa-miR-3944-3p | 1.141 | 3.04E-05 |
| **MIMAT0022292** | hsa-miR-548au-3p | 1.139 | 1.98E-04 |
| **MIMAT0025458** | hsa-miR-6501-5p | 1.139 | 7.41E-03 |
| **MIMAT0027507** | hsa-miR-6803-3p | 1.139 | 9.88E-06 |
| **MIMAT0027495** | hsa-miR-6797-3p | 1.138 | 6.82E-04 |
| **MIMAT0019973** | hsa-miR-4797-3p | 1.137 | 1.84E-03 |
| **MIMAT0019772** | hsa-miR-4685-3p | 1.137 | 7.01E-06 |
| **MIMAT0004950** | hsa-miR-877-3p | 1.135 | 2.73E-03 |
| **MIMAT0022500** | hsa-miR-5706 | 1.132 | 5.21E-05 |
| **MIMAT0027654** | hsa-miR-6877-5p | 1.132 | 7.41E-03 |
| **MIMAT0028220** | hsa-miR-7155-5p | 1.127 | 8.76E-04 |
| **MIMAT0027517** | hsa-miR-6808-3p | 1.126 | 5.15E-05 |
| **MIMAT0019712** | hsa-miR-4649-3p | 1.124 | 2.56E-05 |
| **MIMAT0026623** | hsa-miR-627-3p | 1.122 | 1.10E-06 |
| **MIMAT0027363** | hsa-miR-6731-5p | 1.121 | 2.60E-04 |
| **MIMAT0019000** | hsa-miR-4473 | 1.121 | 4.27E-04 |
| **MIMAT0007400** | hsa-miR-1538 | 1.121 | 1.32E-03 |
| **MIMAT0019724** | hsa-miR-4657 | 1.118 | 8.17E-04 |
| **MIMAT0003323** | hsa-miR-548d-3p | 1.117 | 4.87E-03 |
| **MIMAT0019748** | hsa-miR-219b-3p | 1.116 | 9.15E-03 |
| **MIMAT0027103** | hsa-miR-5699-5p | 1.110 | 1.22E-03 |
| **MIMAT0014987** | hsa-miR-548s | 1.106 | 4.00E-03 |
| **MIMAT0003246** | hsa-miR-581 | 1.106 | 2.21E-03 |
| **MIMAT0015032** | hsa-miR-3158-3p | 1.105 | 1.61E-03 |
| **MIMAT0003336** | hsa-miR-658 | 1.102 | 5.75E-03 |
| **MIMAT0005941** | hsa-miR-1284 | 1.101 | 2.08E-04 |
| **MIMAT0021022** | hsa-miR-5001-3p | 1.101 | 9.64E-03 |
| **MIMAT0011163** | hsa-miR-548q | 1.095 | 4.10E-03 |
| **MIMAT0018112** | hsa-miR-3684 | 1.095 | 2.68E-03 |
| **MIMAT0019943** | hsa-miR-4781-3p | 1.093 | 2.11E-04 |
| **MIMAT0022271** | hsa-miR-664b-5p | 1.092 | 2.44E-03 |
| **MIMAT0003313** | hsa-miR-643 | 1.087 | 5.66E-03 |
| **MIMAT0027505** | hsa-miR-6802-3p | 1.081 | 5.11E-03 |
| **MIMAT0015031** | hsa-miR-3157-5p | 1.076 | 4.26E-03 |
| **MIMAT0020959** | hsa-miR-4536-3p | -1.109 | 9.68E-03 |
| **MIMAT0021117** | hsa-miR-5187-5p | -1.153 | 3.04E-03 |
| **MIMAT0004674** | hsa-miR-30c-1-3p | -1.165 | 5.32E-03 |
| **MIMAT0027513** | hsa-miR-6806-3p | -1.173 | 2.43E-03 |
| **MIMAT0018204** | hsa-miR-676-3p | -1.184 | 3.40E-03 |
| **MIMAT0005914** | hsa-miR-1262 | -1.189 | 3.31E-03 |
| **MIMAT0022706** | hsa-miR-561-5p | -1.191 | 9.36E-04 |
| **MIMAT0026618** | hsa-miR-585-5p | -1.192 | 3.66E-03 |
| **MIMAT0000439** | hsa-miR-153-3p | -1.205 | 6.28E-03 |
| **MIMAT0000083** | hsa-miR-26b-5p | -1.232 | 4.03E-03 |

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| **MIMAT0000734** | hsa-miR-380-5p | -1.238 | 6.80E-03 |
| **MIMAT0000414** | hsa-let-7g-5p | -1.241 | 5.05E-04 |
| **MIMAT0001629** | hsa-miR-329-3p | -1.246 | 6.04E-03 |
| **MIMAT0000273** | hsa-miR-216a-5p | -1.248 | 4.66E-03 |
| **MIMAT0000232** | hsa-miR-199a-3p | -1.257 | 5.52E-03 |
| **MIMAT0004563** | hsa-miR-199b-3p | -1.258 | 5.41E-03 |
| **MIMAT0004681** | hsa-miR-26a-2-3p | -1.262 | 8.94E-04 |
| **MIMAT0003254** | hsa-miR-548b-3p | -1.267 | 1.44E-03 |
| **MIMAT0004955** | hsa-miR-374b-5p | -1.271 | 6.98E-03 |
| **MIMAT0022705** | hsa-miR-539-3p | -1.274 | 9.35E-04 |
| **MIMAT0000090** | hsa-miR-32-5p | -1.278 | 4.05E-03 |
| **MIMAT0000438** | hsa-miR-152-3p | -1.294 | 7.04E-03 |
| **MIMAT0005878** | hsa-miR-1287-5p | -1.310 | 1.64E-03 |
| **MIMAT0000737** | hsa-miR-382-5p | -1.321 | 7.79E-03 |
| **MIMAT0004592** | hsa-miR-125b-1-3p | -1.321 | 4.08E-04 |
| **MIMAT0004688** | hsa-miR-374a-3p | -1.326 | 2.46E-05 |
| **MIMAT0000431** | hsa-miR-140-5p | -1.334 | 1.11E-05 |
| **MIMAT0010214** | hsa-miR-151b | -1.339 | 7.61E-05 |
| **MIMAT0003879** | hsa-miR-758-3p | -1.353 | 2.10E-03 |
| **MIMAT0000062** | hsa-let-7a-5p | -1.354 | 8.06E-05 |
| **MIMAT0004615** | hsa-miR-195-3p | -1.360 | 1.04E-04 |
| **MIMAT0000730** | hsa-miR-377-3p | -1.362 | 1.52E-03 |
| **MIMAT0003266** | hsa-miR-598-3p | -1.364 | 1.68E-04 |
| **MIMAT0000245** | hsa-miR-30d-5p | -1.374 | 3.99E-05 |
| **MIMAT0000437** | hsa-miR-145-5p | -1.386 | 2.65E-03 |
| **MIMAT0003386** | hsa-miR-376a-5p | -1.393 | 2.59E-03 |
| **MIMAT0004515** | hsa-miR-29b-2-5p | -1.394 | 8.15E-04 |
| **MIMAT0004683** | hsa-miR-362-3p | -1.395 | 1.35E-05 |
| **MIMAT0004551** | hsa-miR-30d-3p | -1.398 | 6.23E-11 |
| **MIMAT0000272** | hsa-miR-215-5p | -1.398 | 3.64E-04 |
| **MIMAT0004809** | hsa-miR-628-5p | -1.403 | 4.46E-04 |
| **MIMAT0000447** | hsa-miR-134-5p | -1.411 | 1.27E-03 |
| **MIMAT0000067** | hsa-let-7f-5p | -1.413 | 1.54E-04 |
| **MIMAT0030019** | hsa-miR-7704 | -1.423 | 5.00E-03 |
| **MIMAT0019071** | hsa-miR-4532 | -1.430 | 7.19E-04 |
| **MIMAT0000418** | hsa-miR-23b-3p | -1.436 | 7.29E-06 |
| **MIMAT0003161** | hsa-miR-493-3p | -1.445 | 1.75E-03 |
| **MIMAT0003332** | hsa-miR-656-3p | -1.454 | 4.74E-05 |
| **MIMAT0005948** | hsa-miR-664a-5p | -1.454 | 1.00E-08 |
| **MIMAT0005920** | hsa-miR-1266-5p | -1.473 | 4.13E-04 |
| **MIMAT0004604** | hsa-miR-127-5p | -1.497 | 3.58E-04 |
| **MIMAT0000420** | hsa-miR-30b-5p | -1.522 | 5.65E-04 |
| **MIMAT0000707** | hsa-miR-363-3p | -1.578 | 1.73E-05 |
| **MIMAT0005899** | hsa-miR-1247-5p | -1.597 | 2.87E-03 |
| **MIMAT0000727** | hsa-miR-374a-5p | -1.604 | 1.37E-09 |
| **MIMAT0002818** | hsa-miR-496 | -1.624 | 9.31E-05 |
| **MIMAT0000729** | hsa-miR-376a-3p | -1.642 | 3.27E-04 |
| **MIMAT0004692** | hsa-miR-340-5p | -1.646 | 3.28E-06 |

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|---|---|---|---|
| **MIMAT0000419** | hsa-miR-27b-3p | -1.705 | 1.13E-06 |
| **MIMAT0000094** | hsa-miR-95-3p | -1.708 | 7.15E-03 |
| **MIMAT0003328** | hsa-miR-653-5p | -1.731 | 1.13E-05 |
| **MIMAT0022862** | hsa-miR-381-5p | -1.757 | 7.86E-03 |
| **MIMAT0026472** | hsa-let-7c-3p | -1.771 | 1.15E-06 |
| **MIMAT0000452** | hsa-miR-154-5p | -1.786 | 2.37E-06 |
| **MIMAT0006789** | hsa-miR-1468-5p | -1.827 | 3.61E-09 |
| **MIMAT0004600** | hsa-miR-144-5p | -1.830 | 5.19E-03 |
| **MIMAT0004673** | hsa-miR-29c-5p | -1.842 | 2.68E-06 |
| **MIMAT0005909** | hsa-miR-1258 | -1.845 | 7.52E-07 |
| **MIMAT0000763** | hsa-miR-338-3p | -1.847 | 8.80E-05 |
| **MIMAT0000444** | hsa-miR-126-5p | -1.861 | 1.14E-09 |
| **MIMAT0002820** | hsa-miR-497-5p | -1.880 | 8.35E-12 |
| **MIMAT0004902** | hsa-miR-891a-5p | -1.884 | 4.32E-06 |
| **MIMAT0004690** | hsa-miR-379-3p | -1.886 | 1.39E-05 |
| **MIMAT0004813** | hsa-miR-411-3p | -1.887 | 2.02E-04 |
| **MIMAT0000458** | hsa-miR-190a-5p | -1.895 | 3.22E-08 |
| **MIMAT0004597** | hsa-miR-140-3p | -1.914 | 1.29E-13 |
| **MIMAT0004921** | hsa-miR-889-3p | -1.949 | 3.32E-08 |
| **MIMAT0004947** | hsa-miR-885-5p | -1.957 | 1.84E-03 |
| **MIMAT0000263** | hsa-miR-199b-5p | -1.982 | 1.39E-06 |
| **MIMAT0000423** | hsa-miR-125b-5p | -1.987 | 7.72E-13 |
| **MIMAT0003331** | hsa-miR-655-3p | -1.989 | 3.76E-05 |
| **MIMAT0000275** | hsa-miR-218-5p | -1.999 | 2.90E-09 |
| **MIMAT0026483** | hsa-miR-370-5p | -2.015 | 1.02E-09 |
| **MIMAT0022929** | hsa-miR-758-5p | -2.025 | 1.90E-06 |
| **MIMAT0004549** | hsa-miR-148a-5p | -2.028 | 5.11E-04 |
| **MIMAT0000448** | hsa-miR-136-5p | -2.069 | 8.49E-05 |
| **MIMAT0000693** | hsa-miR-30e-3p | -2.077 | 4.62E-11 |
| **MIMAT0001631** | hsa-miR-451a | -2.146 | 1.08E-03 |
| **MIMAT0002814** | hsa-miR-432-5p | -2.155 | 2.44E-07 |
| **MIMAT0000082** | hsa-miR-26a-5p | -2.173 | 6.35E-12 |
| **MIMAT0000765** | hsa-miR-335-5p | -2.185 | 1.01E-08 |
| **MIMAT0004757** | hsa-miR-431-3p | -2.190 | 5.59E-05 |
| **MIMAT0000692** | hsa-miR-30e-5p | -2.192 | 3.31E-11 |
| **MIMAT0003180** | hsa-miR-487b-3p | -2.220 | 5.46E-08 |
| **MIMAT0018926** | hsa-miR-378d | -2.265 | 1.61E-05 |
| **MIMAT0000274** | hsa-miR-217-5p | -2.266 | 1.89E-06 |
| **MIMAT0004513** | hsa-miR-101-5p | -2.299 | 1.07E-10 |
| **MIMAT0000086** | hsa-miR-29a-3p | -2.309 | 2.75E-14 |
| **MIMAT0000250** | hsa-miR-139-5p | -2.324 | 6.73E-07 |
| **MIMAT0004701** | hsa-miR-338-5p | -2.329 | 1.08E-09 |
| **MIMAT0000721** | hsa-miR-369-3p | -2.329 | 5.55E-08 |
| **MIMAT0002883** | hsa-miR-514a-3p | -2.336 | 6.43E-04 |
| **MIMAT0004601** | hsa-miR-145-3p | -2.337 | 3.02E-13 |
| **MIMAT0004511** | hsa-miR-99a-3p | -2.338 | 2.20E-11 |
| **MIMAT0001621** | hsa-miR-369-5p | -2.409 | 8.33E-09 |
| **MIMAT0004689** | hsa-miR-377-5p | -2.435 | 4.61E-07 |

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|---|---|---|---|
| **MIMAT0002880** | hsa-miR-508-3p | -2.548 | 1.64E-04 |
| **MIMAT0000436** | hsa-miR-144-3p | -2.552 | 2.76E-05 |
| **MIMAT0000731** | hsa-miR-378a-5p | -2.584 | 2.69E-05 |
| **MIMAT0000435** | hsa-miR-143-3p | -2.587 | 3.86E-07 |
| **MIMAT0002817** | hsa-miR-495-3p | -2.600 | 7.34E-07 |
| **MIMAT0026478** | hsa-miR-133a-5p | -2.608 | 2.30E-03 |
| **MIMAT0000446** | hsa-miR-127-3p | -2.885 | 2.97E-09 |
| **MIMAT0004960** | hsa-miR-208b-3p | -2.977 | 2.91E-04 |
| **MIMAT0004814** | hsa-miR-654-3p | -3.031 | 1.43E-09 |
| **MIMAT0000754** | hsa-miR-337-3p | -3.073 | 1.01E-08 |
| **MIMAT0002177** | hsa-miR-486-5p | -3.099 | 1.77E-05 |
| **MIMAT0000720** | hsa-miR-376c-3p | -3.146 | 1.07E-08 |
| **MIMAT0025477** | hsa-miR-6510-3p | -3.149 | 4.10E-05 |
| **MIMAT0000732** | hsa-miR-378a-3p | -3.195 | 2.31E-07 |
| **MIMAT0000098** | hsa-miR-100-5p | -3.215 | 1.59E-19 |
| **MIMAT0002870** | **hsa-miR-499a-5p** | -3.296 | 3.76E-05 |
| **MIMAT0000733** | **hsa-miR-379-5p** | -3.298 | 1.29E-10 |
| **MIMAT0002890** | **hsa-miR-299-5p** | -3.504 | 8.97E-07 |
| **MIMAT0000461** | **hsa-miR-195-5p** | -3.510 | 7.79E-14 |
| **MIMAT0022721** | **hsa-miR-1247-3p** | -3.553 | 3.40E-07 |
| **MIMAT0016847** | **hsa-miR-378c** | -3.670 | 4.61E-08 |
| **MIMAT0002171** | **hsa-miR-410-3p** | -3.684 | 9.33E-12 |
| **MIMAT0004603** | **hsa-miR-125b-2-3p** | -3.694 | 1.52E-18 |
| **MIMAT0004606** | **hsa-miR-136-3p** | -3.797 | 1.08E-12 |
| **MIMAT0004550** | **hsa-miR-30c-2-3p** | -3.881 | 1.03E-12 |
| **MIMAT0004552** | **hsa-miR-139-3p** | -3.937 | 3.02E-14 |
| **MIMAT0000099** | **hsa-miR-101-3p** | -4.017 | 3.64E-23 |
| **MIMAT0000087** | **hsa-miR-30a-5p** | -4.132 | 6.93E-14 |
| **MIMAT0003329** | **hsa-miR-411-5p** | -4.160 | 2.03E-10 |
| **MIMAT0000265** | **hsa-miR-204-5p** | -4.519 | 1.28E-17 |
| **MIMAT0000681** | **hsa-miR-29c-3p** | -4.539 | 5.24E-17 |
| **MIMAT0000064** | **hsa-let-7c-5p** | -4.674 | 3.68E-22 |
| **MIMAT0000462** | **hsa-miR-206** | -5.228 | 4.62E-03 |
| **MIMAT0000736** | **hsa-miR-381-3p** | -5.293 | 5.06E-08 |
| **MIMAT0000770** | **hsa-miR-133b** | -5.580 | 3.66E-04 |
| **MIMAT0000088** | **hsa-miR-30a-3p** | -5.696 | 2.66E-13 |
| **MIMAT0000097** | **hsa-miR-99a-5p** | -5.746 | 1.85E-27 |
| **MIMAT0000427** | **hsa-miR-133a-3p** | -7.055 | 2.93E-04 |
| **MIMAT0000416** | **hsa-miR-1-3p** | -10.663 | 8.80E-06 |
| **MIMAT0000728** | **hsa-miR-375-3p** | -18.183 | 1.33E-11 |

** In bold the 50 most up-and down-regulated miRs;*p-values were obtained with Student's t-test*

**Table S4.2** mirDIP number of prediction source and interaction levels between the 11-oral cancer-correlated miRs and the genes selected by COSMIC.

| Genes | miRs | hsa-miR-196a-5p | hsa-miR-503-5p | hsa-miR-18a-5p | hsa-miR-196b-5p | hsa-miR-195-5p | hsa-miR-99a-5p | hsa-miR-379-5p | hsa-miR-133a-3p | hsa-miR-411-5p | hsa-miR-1-3p | hsa-miR-375-3p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TP53 (43 %)** | **Int. Score** | 0.359 | 0.131 | 0.062 | 0.167 | 0.195 | 0.027 | 0.397 | 0.124 | 0.028 | 0.118 | 0.203 |
| | **N. Sources** | 6 | 5 | 4 | 6 | 7 | 3 | 10 | 4 | 3 | 5 | 7 |
| **FAT1 (28 %)** | **Int. Score** | 0.157 | 0.179 | 0.042 | 0.154 | 0.072 | 0.122 | 0.032 | 0.147 | 0.020 | 0.346 | 0.193 |
| | **N. Sources** | 5 | 5 | 4 | 4 | 4 | 4 | 3 | 5 | 2 | 8 | 5 |
| **CASP8 (23 %)** | **Int. Score** | 0.274 | 0.057 | 0.062 | 0.271 | 0.038 | 0.020 | 0.104 | 0.140 | 0.137 | 0.056 | 0.168 |
| | **N. Sources** | 8 | 4 | 4 | 8 | 3 | 2 | 6 | 5 | 5 | 4 | 7 |
| **TERT (22 %)** | **Int. Score** | 0.018 | 0.125 | 0.059 | 0.024 | 0.279 | 0.020 | 0.020 | 0.156 | 0.020 | 0.039 | 0.018 |
| | **N. Sources** | 2 | 5 | 5 | 2 | 6 | 2 | 2 | 7 | 2 | 3 | 2 |
| **NOTCH 1 (20 %)** | **Int. Score** | 0.177 | 0.092 | 0.146 | 0.222 | 0.123 | 0.037 | 0.043 | 0.055 | 0.028 | 0.035 | 0.151 |
| | **N. Sources** | 5 | 4 | 5 | 6 | 4 | 3 | 4 | 3 | 3 | 3 | 4 |
| **CDKN2A (16 %)** | **Int. Score** | 0.144 | 0.180 | 0.046 | 0.147 | 0.430 | 0.040 | 0.061 | 0.027 | 0.020 | 0.097 | 0.029 |
| | **N. Sources** | 5 | 7 | 4 | 5 | 10 | 3 | 4 | 3 | 2 | 5 | 3 |
| **HRAS (10 %)** | **Int. Score** | 0.017 | 0.114 | 0.019 | 0.023 | 0.054 | 0.019 | 0.020 | 0.024 | 0.020 | 0.020 | 0.036 |
| | **N. Sources** | 2 | 4 | 2 | 2 | 4 | 2 | 2 | 3 | 2 | 2 | 3 |
| **KMT2D (10 %)** | **Int. Score** | 0.206 | 0.430 | 0.440 | 0.222 | 0.739 | 0.156 | 0.041 | 0.126 | 0.020 | 0.120 | 0.200 |
| | **N. Sources** | 6 | 11 | 10 | 9 | 17 | 5 | 4 | 4 | 2 | 3 | 4 |
| **FGFR3 (8 %)** | **Int. Score** | 0.159 | 0.253 | 0.292 | 0.140 | 0.211 | 0.907 | 0.044 | 0.092 | 0.019 | 0.156 | 0.019 |
| | **N. Sources** | 7 | 13 | 11 | 6 | 10 | 21 | 4 | 5 | 2 | 5 | 2 |
| **PIK3CA (8 %)** | **Int. Score** | 0.036 | 0.032 | 0.066 | 0.042 | 0.167 | 0.011 | 0.068 | 0.123 | 0.011 | 0.057 | 0.279 |
| | **N. Sources** | 3 | 2 | 3 | 3 | 4 | 1 | 3 | 3 | 1 | 3 | 7 |

**Table S4.3** TCGA HNSC gene expression values of the 10 miR interacting genes.

| Genes | FC Tumor vs Normal | p-value* |
|---|---|---|
| **TP53** | -1.383 | 1.79E-04 |
| **FAT1** | 2.301 | 2.55E-06 |
| **CASP8** | 1.713 | 2.55E-06 |
| **TERT** | 3.842 | 2.54E-13 |
| NOTCH1 | -1.155 | 2.11E-01 |
| **CDKN2A** | 3.001 | 2.57E-05 |
| HRAS | 1.259 | 6.17E-02 |
| MLL2 | -1.080 | 3.90E-01 |
| FGFR3 | 1.374 | 2.94E-01 |
| **PIK3CA** | 1.455 | 9.99E-06 |

*In bold the significantly altered genes;*p-values were calculated by Student's t-test*

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