UNIVERSITA' DEGLI STUDI DI CATANIA
DEPARTMENT OF BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES
International Ph.D. in Basic and Applied Biomedical Sciences
XXXIV Cycle

Ph.D. Thesis

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Liquid Biopsy for the Early Diagnosis of Oral Cancer: Role of microRNAs as Novel Biomarkers

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ACADEMIC YEAR 2020-2021

ABSTRACT

Oral cancer is the most frequent and aggressive tumor of the oral cavity. Due to its highly aggressive behavior, oral cancer represents a serious problem for human health. In addition, despite the availability of screening programs and the accessible anatomical sites of oral lesions, the incidence, prevalence and mortality rates are high as the diagnosis is often made when the neoplasm is in an advanced state. Therefore, advanced oral cancer patients have often a poor prognosis. Therefore, it is evident how the discovery of novel biomarkers for the management of this pathology is necessary to early detect oral cancer and improve patients' clinical outcomes.

At present there are no effective diagnostic or prognostic biomarkers for oral cancer, on this basis, the aim of the present study was to identify and validate a set of microRNAs (miRNAs) involved in the development and progression of oral cancer thus useful as predictive biomarkers for this tumor. For this purpose, both computational and experimental analyses were performed in order to establish the diagnostic and prognostic potential of the miRNAs here identified. Through the analysis of miRNA expression data and clinical data obtained from The Cancer Genome Atlas Head and Neck Cancer (HNSC) and GEO DataSets databases a set of eleven miRNAs significantly involved in oral cancer was identified. Through further computational analyses, the functional roles of these 11 miRNAs were evaluated and four miRNAs, the up-regulated hsa-miR-196a-5p and hsa-miR-503-5p, and the two down-regulated miRNAs hsa-miR-133a-3p and hsa-miR-375-3p, were selected for the validation analyses performed on liquid biopsy samples collected from oral cancer patients, individual at risk for this tumor and healthy controls.

The validation analyses on liquid biopsy samples collected from patients and controls were performed by using the high sensitivity droplet digital PCR (ddPCR) amplification system in order to evaluate the expression levels of selected miRNAs in serum and saliva samples obtained from oral cancer patients, oral lichen planus (OLP) patients and healthy controls.

The ddPCR results obtained in serum samples revealed the high diagnostic and predictive value of hsa-miR-503-5p and hsa-miR-133a-3p. Of note, the diagnostic potential of miRNAs was higher in saliva samples which showed a higher significant difference in the expression levels of both hsa-miR-503-5p and hsa-miR-133a-3p. More in detail, by analyzing the miRNA expression levels of hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p in normal individuals, OLP and oral cancer patients the expression levels of hsa-miR-503-5p observed in both serum and saliva samples were strongly predictive for OLP and oral cancer development. Also in this case, saliva samples were more reliable compared to serum samples in correctly diagnosing oral lesions. Further statistical analyses confirmed the high diagnostic value of the miRNAs identified. Also the prognostic value of the selected miRNAs was confirmed with further computational analyses.

Overall, the results obtained revealed that the evaluation of hsa-miR-196a-5p, hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p in serum and saliva samples of patients with suspicious lesions may be helpful for the early identification of oral cancer. Therefore, the use of ddPCR and liquid biopsy samples may represent an innovative strategy to improve the management of oral cancer.

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1. INTRODUCTION

The first portion of the digestive and respiratory tract is the oral cavity that is involved in the ingestion and chewing of food, as well as phonation and ventilation.

The incidence of malignant lesions involving this area is quite frequent, about 10% of malignant tumors of the human body due to the abnormal proliferation of oral mucosa cells subjected to genetic damage. Precancerous lesions, such as leukoplakias, erythroplasia, lichen, submucosal fibrosis, Fanconi anemia represent the early manifestations of the oral cavity carcinomas (15–40%).

The most frequent malignant tumor of the oral cavity is the Oral Squamous Cells Carcinoma (OSCC) that originates from the epithelium lining the oral cavity and can occur in any area of the oral cavity. It is responsible for 3% of death in men and 1% in women and shows a 5-year survival rate of 66% in the absence of metastases at diagnosis and only 9% if distant metastases are present. Because of the absence of effective cancer screening programs, the diagnosis of OSCC is performed when the lesions are already symptomatic in late stages. For these reasons, it is essential to promote the early detection of oral cancer. Furthermore, the treatment of advanced OSCC is challenging, related to the need for radical surgery and reconstruction in such complex anatomical district, associated with the general condition of the patients. Generally, treatment oflow-gradee OSCC can involve minor surgery associated with medical therapy or radiotherapy.

1.1 Epidemiology

1.1.1 Incidence and prevalence

Oral cancer is one of the most frequent cancers worldwide, accounting for about 354,864 new diagnoses and about 177,384 new deaths per year (Bray, F., et al. 2018). The American Cancer Society estimated that 53,260 new cases (38,330 men and 14,880 women) were diagnosed in the United States in 2020, with around 10,750 deaths in both sexes. These data show an increase in the incidence of this tumor (Siegel R.L., et al. 2020). The prevalence and incidence of oral cancer differ, depending on sex, age and region (Kevin Shield et al. 2012), and

the frequency of oral cavity cancer is very high in some countries including India (associated with the use of non-smoking tobacco and betel quid) (Shield K.D., et al. 2016). Due to the alcohol consumption, tobacco smoke and human papillomavirus infection, incidence of oropharynx cancer is also high in North America and Europe, particularly in Hungary, Slovakia, Germany, and France, mainly associated with (Amarasinghe, et al. 2019) (Figure 1).

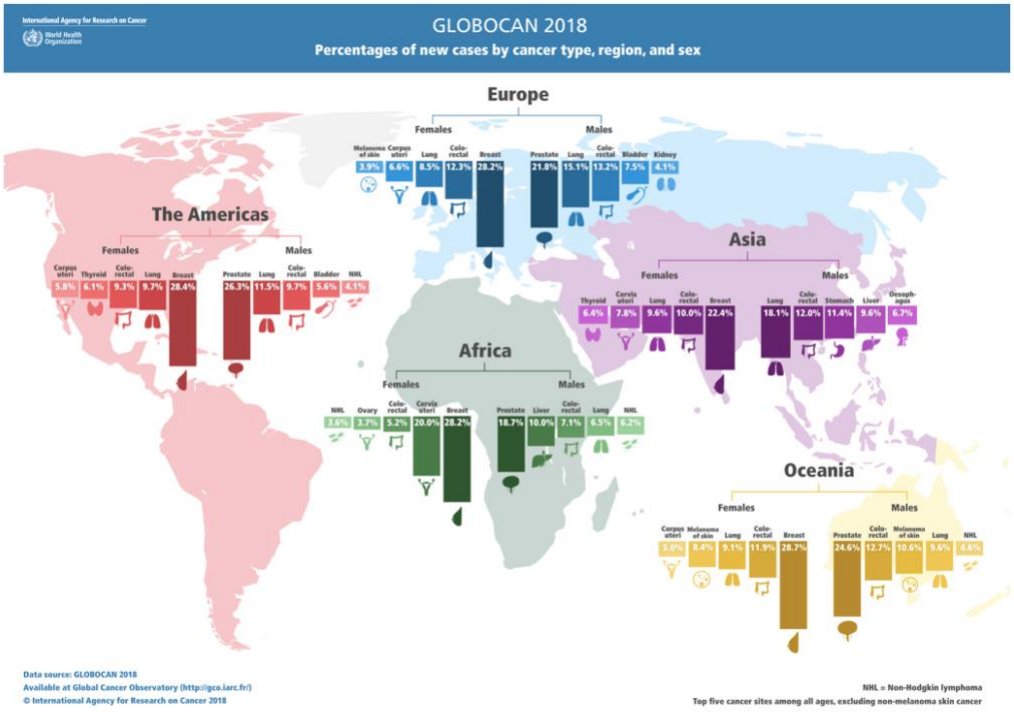

Figure 1. GLOBOCAN 2018: Percentages of new cases by cancer type, region, and sex.

1.1.2 Age and sex

The incidence of oral cancer varied significantly according to age and the Human Development Index (HDI), which is a comparative index of the development of different countries, calculated considering the different rates of life expectancy, education and gross national income per capita, which has become a standard tool for measuring a country's well-being. Women in low-HDI countries, particularly in the South-Central Asian region, where this cancer is common, have the highest proportional incidence. It should be pointed out that the incidence of oral cancer is constantly increasing among men rather than women, with an M:F ratio of 2:1, which goes from 5:2 for Central and Eastern Europe to 1:4 for North Africa, West Asia, and Oceania (Shield, K.D., et al. 2017). This

difference reflects the incidence related to sex, tobacco habit and alcohol that are highest in men. However, this trend is becoming the opposite in some countries where women have taken on these habits. The incidence also increases with age, with most cases between 60-65 years. Despite these data, towards the end of the last century, several reports showed a tendency, which seems to continue, for this type of cancer to affect young people (under 45 years of age) (Llewellyn CD, et al. 2001).

1.1.3 Socioeconomic Status and Ethnicity

Oral cancer is linked to a low socio-economic status. Indeed, the highest incidence rates are displayed by the most disadvantaged portions of the population. Conway et al. published in a systematic review of 41 publications, that the risk of occurrence of OCC is strictly associated, especially in men, by low levels of education and income or employment (Conway DI, et al. 2008).
There are several patterns that can be prepossessed the incidence and mortality of oral cancer, mostly explained by different cultural practices related to lifestyle and eating habits. Literature data showed that depending on the region, such as East and South Asia, population has increased rates of oral cancer due to betel quid chewing. It has also been shown that in the United States black males have a higher percentage of oral cancer occurrence than Caucasian or Hispanic males. (Morse DE, Kerr RA 2016). Although there is no clear correlation between the onset of oral cancer in relation to ethnicity, a possible explanation for these differences can be identified in the environmental and socio-economic differences, which affect the lifestyle of different populations all over the world.

1.1.4 Trend and Survival rate

It has been calculated that over the last decade the rate of subjects affected by oral cancer has undergone a significant increase in European countries. Consequently, the overall incidence of OCC is projected to increase due to demographic changes by 62% representing approximately 856,000 new cases per year by 2035. (Shield KD, et al. 2017). In this estimate, despite the progress of surgical techniques and adjuvant and neoadjuvant therapies, there were no significant improvements in terms of 5-year survival rates for oral cancer in the

countries studied. Furthermore, it must be considered that the reference value of survival at 5 years from the diagnosis of the disease is a factor commonly used to indicate the incidence of the disease in the population.

Looking at epidemiological data, in most South Asian countries, it appears that 5-year survival rates are less than 50%, while in the United States, 5-year survival rates for all races have been recorded at 66%. A study, performed between 1998 and 2006, for example, shows how the 3-year overall survival rate increased from 18% to 34% in patients with advanced disease. It is clear that the data emerging from the literature demonstrate a significant reduction in mortality in patients with oral cancer over the last few decades. This improvement is attributed by the authors to the introduction of new adjuvant chemo-radiotherapy treatments for the treatment of patients with advanced tumors. Overall survival rates are primarily dependent on specific factors, such as staging, but also on patient-related factors such as age and comorbidity. The survival rate among young people is significantly higher than in older patients and this may be linked to a lower incidence of comorbidities.

1.2 Risk Factors

There are several factors that positively and negatively influence the incidence of oral cancer and consequently the mortality rates. Eating habits and lifestyles are certainly influencing factors that can be intercepted through a broad prevention campaign. It is now known that alcohol consumption and tobacco represent the two major elements recognized as predisposing factors for the onset of oral cancer.

1.2.1 Modifiable risk factors

The exposure of the individual to environmental agents with oncogenic potential is a clear predisposing factos for the incidence of the onset of the disease, of all those most to be noted are:

- Tobacco smoking: the correlation between the increased onset of OSCC in patients with tobacco smoking has been widely debated in the literature. It is estimated that 80% of people with this cancer have a history of cigarette smoking

(10). The risk of developing cancer increases approximately 6 times in smokers of more than 20 cigarettes per day and is dose-dependent (11).

Smokers with more than 20 cigarettes seem to be twice as risky as smokers with less than 20 cigarettes (12).

Despite the low percentage of cigar and pipe smoking patients with OSCC due to a lower spread, they have a higher risk than cigarette smoking of developing the disease. The resumption of smoking in subjects already surgically treated for squamous carcinoma of the oral cavity increases, in consideration of the staging, from 2 to 6 times (13).

The etiopathogenetic mechanism underlying tissue metaplasia is directly caused by the irritative stimulus directly caused by cigarette smoke on the lining epithelium of the oral cavity, and indirectly due to the release of carcinogenic substances (at least 40 in cigarette smoke), including benzopyrene, free radicals such as hydroquinone and N-nitrosamines (14). The consumption of non-smoked tobacco (chewed tobacco and snuff, which consists in maintaining the quantity of cut tobacco in the labial fornix, between gum and lip) is very relevant, especially in relation to the region to which it belongs. onset of OSCC.

Furthermore, in some countries, it is common to chew tobacco mixed with other substances, which could increase its carcinogenic effect. This is the case of quid, a blend of tobacco, areca nuts, limes and other spices wrapped in a betel leaf. The practice of chewing quid is widespread in India and Southeast Asia and the risk of developing OSCC in quid users is 8% (15).

- Alcohol consumption: Of all people with OSCC in the United States, about one-third are heavy alcohol users (more than 100 grams of alcohol per day), who are 30 times more likely to develop oral and oropharyngeal cancer (16). The pathogenetic mechanism underlying it, as well as for cigarette smoke, is related to the dose taken by the patient. Literature data show that smoking and tobacco, independent risk factors, when combined determine a synergistic, non-additive effect, which is potentiating, consequently determining an increase in incidence of 6-15 times in consumers of both (17). There are no scientific data to support the direct mutagenic action of ethanol; therefore, the carcinogenic action of alcoholic beverages would be due to indirect mechanisms. Alcohol

could act as a solvent for other carcinogens, producing an alteration of morphology characterized by epithelial atrophy, which in turn leads to easier penetration of carcinogens into the oral mucosa (18). Acetaldehyde, responsible for the carcinogenic action of alcohol, is its main metabolite whose transformation is mainly carried out by the enzyme alcohol dehydrogenase (ADH). Acetaldehyde is oxidized to acetate by means of aldehyde dehydrogenase (ALDH). Acetaldehyde causes DNA damage by interfering with DNA synthesis and repair; it also inhibits the 6-methylguanitransferase enzyme responsible for repairing lesions caused by alkylating agents (19). The increase in acetaldehyde accumulation in the body is considered deleterious. The accumulation can occur due to increased activity of ADH enzyme activity, which is present in oral microflora and in the oral mucosa or alternately, reduction in ALDH enzyme can also lead to accumulation of acetaldehyde. Genetic polymorphisms that have been reported in these two enzymes, ADH and ALDH, have been related to the increased risk of alcohol-related cancers, such as oral cancer (20).

- Diet and nutrition: The development of food-related oral cancer has been proven by many epidemiological and laboratory studies. The working group of the International Agency for Research on Cancer (IARC) said that low consumption of fruit or vegetables predisposes to an increased risk of developing cancer. Conversely, the more frequent consumption of fruits and vegetables, particularly carrots, fresh tomatoes, and green peppers, was associated with a reduced risk of oral and pharyngeal cancer. The reason is for a particular nutrient that these foods contain, like vitamin A and related carotenoids (in particular □carotene), vitamin C, vitamin E and selenium, thanks to their powerful antioxidant activity, seem to play a leading role in the protective action against epithelial cancer. Antioxidants, in fact, act by reducing the number of free radicals that could cause DNA mutations or changes in membrane lipid peroxidation. The fundamental role that micronutrients play is determined by their involvement in the modulation of the metabolism of carcinogens, in the maintenance of correct cell differentiation, in the inhibition of cell proliferation and expression of oncogenes, as well as in the maintenance of the correct

functions of the immune system and in the inhibition of the formation of endogenous carcinogens. Literature data show that studies performed on the use of carotene have been conducted in oral cancer and have shown considerable success rates even if a direct cause-effect relationship has not been elucidated. (21).

• Viral infections: Malignant transformation of epithelial cells can be caused by a viral infection. Some viral genes are proto-oncogenes which become oncogenes when inserted into the host's DNA and affect cell growth and proliferation. The main viruses implicated in the development of oral cancer are the Epstein-Barr virus (EBV), the human papilloma virus (HPV) and the herpes simplex virus (HSV). Precancerous lesions such as oral hairy leucoplakia and "lymphoproliferative disease" in immunosuppressed patients are related to EBV infection. The relationship of EBV to oral squamous cell carcinoma (OSCC) is unclear. Prevalence studies have shown the presence of EBV in OSCC patients but do not demonstrate a causal association. The association of EBV and the cause of salivary gland carcinoma, often referred to as malignant lymphoepithelial lesion. Another study demonstrated the presence of EBV DNA in a submandibular gland adenocarcinoma in a Finnish child. (22). The most frequent viral infection related to the onset of OSCC is that associated with HPV. HPVs are DNA viruses of the papillomaviridae family, epitheliotropic viruses, especially for squamous epithelia. To date, over 100 viral genotypes have been characterized which, in relation to their oncogenic potential, have been divided into two groups: High Grade HPV (HR HPV; 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59, 66, 70, 82) associated with potentially malignant lesions and low-grade HPV (LR HPV; 2,4, 6, 11, 13, 32, 42, 43, 44, 54, 61, 70, 72, 81) most commonly associated with benign manifestations (vulgar warts, warts, focal epithelial hyperplasia, squamous cell papilloma’s, Bowen's papillomatosis). The role of HPV in cancer development is that its genes and gene products can disrupt the cell cycle mechanism. The oncogenetic mechanism manifests itself through the HPV-derived protein transcripts, in particular the HPV HR E6 and E7 oncoproteins, which interact with host cells to influence epithelial differentiation and inhibit the regulatory function of p53 and tumor suppressor pRb in cell

replication, DNA repair and apoptosis. HPV has been detected in OSCC, dysplasia and other benign lesions using various techniques and some studies have shown the presence of HPV in normal oral mucosa, making the role of HPV in oral carcinogenesis speculative (23). The frequent finding of the HPV16 strain in genital and oral cancers clearly indicates the possible source of HPV infection in the oral cavity. It has been proposed that the role of HSV in carcinogenesis is the enhancement of the activation, amplification of the overexpression of pre-existing oncogenes such as c-myc and c-erb-B-1, based on the evidence from in vitro studies (24).

- Fungal infection: In the field of fungal infections related to the pathogenesis of precancerous lesions, those caused by Candida albicans have been implicated as the most frequent. Superficial fungal hyphae of Candida albicans have been identified overlapping leucoplakia, particularly nodular leukoplakia, many of which have undergone malignant transformation. habitual commensals in the oral cavity, Candida species become opportunistic during host immunosuppression due to systemic disease or drug therapy. In addition to immunocompromised individuals, Candida infection may coexist or be associated with other risk factors such as in chronic smokers which may be synergistic in the development of oral cancer. There is evidence that Candida possesses enzymes needed by food substances to produce nitrosamines and chemicals that have been implicated in carcinogenesis (25).

1.2.2 Non-modifiable risk factors

Taking into consideration all those non-modifiable factors that influence the onset of OSCC, the genetic susceptibility, or predisposition, to oral cancer certainly appears relevant, especially in young cases, presumably based, as in the case of other types of tumors, on the transmission of defects on the part of genes involved in the processes of carcinogenic metabolism, DNA repair and control of the cell cycle.

Literature data highlights the existence of a significant family component in the development of oral cancer. The estimated risk of developing neoplasms in first-degree relatives with oral cancer ranges from 1.1 (26) to 3.8 (27), although some of these cancers generally refer to the head and neck area. In a small percentage

of patients with OSCC, it was also possible to detect the presence of family aggregations probably with an autosomal dominant inheritance pattern (28). The polymorphisms of the genes coding for detoxifying enzymes (CYP1A1 and GSTM1) were positive in the allelic association test (29) (30), therefore these data support the idea that there are genetic factors that can increase the concrete risk of onset of OSCC. Possible polymorphisms have also been identified in genes involved in cell cycle control. For example, a SNP in the CCND1 gene, which codes for cyclin D, was found to be associated with the onset of oral cancer.

Squamous cell carcinoma of the oral cavity has finally been associated with some genetic syndromes such as dyskeratosis congenita and Fanconi anaemia. Patients with the latter, an autosomal recessive disease characterized by congenital anomalies and bone marrow defects, show a certain predisposition to the development of tumors, in particular squamous cell carcinoma of the oral cavity and anogenital regions (31). Berkower and colleagues in 1988 (32) also observed an increased susceptibility to oral cancer in patients with Bloom syndrome (growth retardation and predisposition to developing different types of cancer).

1.2.3 Other factors

Several other risk factors have been related to the onset of OSCC. More local and systemic conditions that can favor the onset of cancer and are identified as cofactors as they cause a weakening of the mucous membranes, damaging them and allowing a more rapid evolution from precancerous lesion to carcinoma in situ, up to the development of a real neoplasm invasive.

Alterations of the oral state such as malocclusions, fillings or incongruous prostheses, poor oral hygiene, poor dental condition such as sharp / fractured teeth due to caries / trauma and chronic ulceration from a poorly adapted prosthesis can favor the establishment of mechanisms of alteration and metaplasia of the oral mucosa. About 8.9% of oral cancer cases appear to be related to poor oral hygiene, especially if there are ≤ 2 indices of good oral hygiene: not wearing prostheses, not having gum disease (or bleeding), <5 missing teeth, brush your teeth at least once a day and visit the dentist at least

once a year. The data in the literature show that the presence of repeated microtraumas and a state of inflammation are the cause of the alterations (33).

States of immunosuppression, congenital or acquired: The defence of the organism against everything that can be harmful is entrusted to the immune system, so it is able to recognize what is "self" and what is "not self". Non-self-antigens can be pathogenic, but they are also expressed on cancer cells. This is thought to be due to genetic and epigenetic modifications occurring in cancer cells, which then lead to the expression on the cytoplasmic membrane of altered proteins which are then recognized by the immune system as abnormal and therefore harmful. Immunodeficiency disorders can be primary or secondary. When secondary they are associated with systemic disorders, such as diabetes, hypo-nutrition, or HIV infection, or are determined by immunosuppressive therapy. This treatment is mainly used in the case of organ transplantation, to prevent the immune system from attacking the organ and causing rejection. Immune system deficient conditions not only prevent the immune system from attacking cancer cells, but also facilitate infection with oncogenic viruses. This episode can be considered a cofactor in the development of a tumor pathology. Based on the hypothesis of the possible direct or indirect oncogenicity of chemotherapy and immunosuppressive therapy, studies have been carried out on patients transplanted under immunosuppressive treatment, the results of which show the appearance of a de novo tumor in 5-6% of cases, and it is assumed that it may be cancer cells transmitted by the donor during organ transplantation. The suppression of the immune system prevents the body from attacking the harmful cells, allowing them to replicate and grow within the transplanted organ. In patients who were previously affected by neoplasia, metastases occurred in 41% of cases and a new tumor in 6% of cases. It is doubtful, therefore, that chemotherapy can, on the one hand, control tumor growth, but on the other hand promote the appearance of new tumors (34).

1.2.4 Molecular alteration

Several molecular alterations associated with these well-known risk factors determine the development of oral cancer.

The influence of key genes involved in the regulation of cellular processes, such as the cell cycle, cell proliferation and apoptosis. The most frequent genetic alterations found linked to OSCC affect TP53, NOTCH1, CDKN2A, SYNE1, PIK3CA, as well as genes related to the EGFR pathway (including TGF-β, fibroblastic growth factor-BP (FGF-BP) and MMK6) (35). the most commonly mutated gene is the TP53 gene, located on chromosome 17, is found in human malignant tumors, and has many important biological functions, including the control of the cell cycle checkpoint, in fact the activation of the p53 protein as a transcription factor that initiates a program of cell cycle arrest, cell senescence or apoptosis. The alteration of the cell cycle regulation pathway is the most altered in oral cancer. The Notch signaling pathway is thought to play important roles in regulating normal cell differentiation and survival. Several human diseases, including cancers and developmental disorders, are caused by the dysregulation of the Notch signal. It is noted that Notch signaling has both oncogenic andtumor-suppressivee roles depending on the cellular context. NOTCH1 was the second most mutated gene in OSCC patients. Many of these missense mutations also occurred at or near important domains such as ligand binding domains (EGF-like domain) (36).

The mechanism based on the control of modifications h

Genetics over the past few decades has shifted towards considering a more comprehensive picture in which DNA methylation, histone modifications and nucleosome placement are now considered a crucial role. An increasingly emerging role is given to the expression of non-coding RNAs (ncRNAs), in particular microRNAs (miRNAs), which can be influenced and at the same time are also influencing epigenetic mechanisms. Irimie et al., In a review, demonstrated that epigenetic modifications, including intragenic / promoter methylation and microRNA (miRNA) deregulation, have been linked to the development of oral cancers by mediating alteration of cellular homeostasis and of physiological processes (37).

1.3 Potential Malignant Disorder

Potentially malignant conditions, or better defined oral precancerous conditions, constitute about 60-80% of the first clinical manifestation of OSCC (38). The

typical clinical manifestation consists of the appearance of whitish and/or red dyskeratotic areas. In this case, the early interception of the patient is of great importance, thanks to a progressive improvement of the prevention campaigns that have taken place over the last few decades, which has led to a progressive reduction in morbidity and mortality for OSCC. Since 2005, the World Health Organization (WHO) has clarified the definition of precancerous lesions by defining and classifying all oral lesions with a predisposition to malignant transformation (39). Defined as morphological alterations of the oral mucosa, potentially malignant disorders constitute the areas in which the onset of an OSCC is much more frequent than the surrounding healthy tissue, preferring the term PRD to "preneoplastic" lesions.

There are several lesions belonging to this group, although the most represented ones include leucoplakia, with all its clinical variants, Oral Lichen Planus (LPO) and also submucosal fibrosis, although the latter is typical only of eastern populations where the chewing of quid leaves.

1.3.1 Erytroplakia

Generally manifested as an asymptomatic lesion, sometimes minimally bleeding, erythroplakia is a red lesion, not attributable to a known cause, of variable size, often even during the same day as it is dependent on blood flow. Factors that potentially determine a protective mechanism from the onset of these lesions are undoubtedly the eating habits, the consumption of fruit and vegetables, for example, is a protective factor, while the consumption of tobacco, alcohol or betel are risk factors for the onset of this lesion in the oral cavity. Furthermore, HPV has also been considered as a possible cofactor in its etiopathogenesis.

From a histological point of view, in most cases there is medium or severe dysplasia and foci of invasive and/or microinvasive carcinoma may be present.

The differential diagnosis with all those areas where inflammation is present following trauma or microtrauma or poor oral hygiene plays a fundamental role in the identification of these lesions. A correct diagnosis is useful and essential for a timely intervention on the lesion.

Literature data state that erythroplakia, as well as leucoplakia, may not necessarily arise de novo, but rather have a precursor, such as lichenoid lesions of the oral mucosa (40).

1.3.2 Leukoplakia

The new definition of Leukoplakia is due to Shanbhag. In fact, in 2007 the same author proposed a new definition for leukoplakia as: - "a predominantly white, irreversible, non-scrapable lesion of the oral mucosa that cannot be characterized clinically or histopathologically like any other lesion / disease and has a higher risk of onset of cancer than its normal counterpart and is usually associated with the consumption of tobacco, betel quid and alcohol, but otherwise may be idiopathic in nature "(41). Therefore, the role of the anamnesis as well as of the diagnosis of exclusion in the identification of the patient with leukoplakia appears central, the objective clinical examination and the histological examination allow to obtain a differential diagnosis. There are several lesions that can enter into differential diagnosis with leukoplakia, they are related to normal anatomical variations such as Fordyce's spots, chewing trauma such as chemical burn or friction keratosis, infectious causes such as oral candidiasis or oral hairy leukoplakia, immunological causes such as psoriasis, lichenoid reaction linked to lupus erythematosus, lichen planus and others.

Data reported in the literature show that the prevalence of leukoplakia is between less than 1% and 5% with a higher percentage in the male sex and an age of onset around 40 years. The data on the percentage of malignant transformation, on the other hand, are not so homogeneous, ranging from 0.3% to 38% and an annual transformation rate of about 1% (42).

Localization and symptoms: the most frequent sites where it is possible to highlight leukoplasic lesions is undoubtedly the region of the genetic and retrocommisural mucosa, even if the same can manifest itself in any part of the mucosa of the oral cavity. The less frequent areas, but with a higher rate of malignant transformation, are those of the oral floor and lingual margins (43). These lesions have the characteristic of manifesting themselves individually or in multiple ways with various sizes and shapes. There are two clinical types of leukoplakia, the homogeneous variant, characterized by a white color distributed

evenly in relation to the surrounding mucosa, and the non-homogeneous variant, in which the non-homogeneous white and red areas can be fragmented, with the possible presence of areas of erosion. Rare clinical variant of red color, but with a very high risk of neoplastic evolution is instead erythroplakia (44). Another type of non-homogeneous lesion is verrucous leukoplakia whose color is homogeneous, but the surface is raised and warty (45). another subtype of verrucous leukoplakia is Proliferative Leukoplakia Verrucosa, characterized by diffuse lesions, (46) resistant to therapy and at high risk of malignant transformation (47). Melanin can leak from cells; it can also give a gray color to some leukoplakia lesions. Of all those described, non-homogeneous lesions are those with the highest risk of malignant transformation, although all leukoplakias should be biopsied to identify their subtype and staging.

Histology: From a histological point of view, leukoplakia does not have a specific appearance, but it is possible to observe different microscopic pictures, from a simple hyperkeratosis to carcinoma precursors (dysplasia) and carcinoma in situ. The presence of dysplasia is still the most affable parameter to identify lesions at risk of malignant transformation, although the absence of dysplasia cannot define a lesion as not at risk (48). the transformation rates of lesions with dysplasia are between 16-36% (49). The presence of moderate to severe dysplasia can double the risk of malignant transformation.

There are several characteristics of epithelial dysplasia involved in leukoplakia Cellular Pleomorphism, in which cells are of abnormal and different shapes; nuclear Atypias, there may also be more prominent nucleoli.

The increase in the number of cells observed in the phase of mitosis, including normal and abnormal mitoses, Loss of the normal organization of the epithelial layers. The distinction between the epithelial layers can be lost. The normally layered squamous epithelium shows progressive changes in cell shape from the basal to the superficial layers, with cells becoming flattered ("scales") towards the surface as a continuous maturation process. In dysplastic epithelium, cells can be oriented vertically instead of flattening towards the surface.

The defining parameters of dysplasia are operator dependent, as pathologists can describe it as mild, moderate, or severe dysplasia. The pathologist must have

experience in this regard as a not short learning cure is necessary. It has been shown that there is a high degree of interobserver variation and poor reproducibility in how dysplasia is classified (51). The term severe dysplasia is assimilated to the term carcinoma in situ, which denotes the presence of neoplastic cells that have not yet penetrated the basement membrane and have invaded other tissues.

1.3.3 Oral Lichen Planus

Chronic mucocutaneous disease, Lichen planus is determined by an autoimmune cell-mediated pathogenic mechanism against the cells of the basal layer (52).

Being one of the most common diseases of the oral mucosa, Oral Lichen Planus (OLP) has a prevalence in the population of about 1-2%, without particular distinctions or racial predilections, but with a prevalence of female sex (53). There is not much evidence of skin involvement, usually self-limited, while oral lesions are chronic and rarely go into remission.

Lesions of oral OLP in the literature are identified as the result of numerous and different pathological manifestations with the same pathogenetic mechanism and with important differences in the etiological agent (54).

These are lesions with very varied clinical features (white, red, ulcerative, and more rarely boiled) associated with histological alterations that are identified in the lichen infiltrate, that is, an inflammatory infiltrate mainly lymphocytic localized below the membrane (55).

The lesions generally present with a bilateral and symmetrical distribution, which suggests the presence of a self-altered immunity and highlights factors that intervene within the oral cavity in its entirety. The presence of these lesions identifies the picture of classic OLP and seems to be the consequence of an extreme hyper-reactivity of the patient towards unknown agents located in the oral cavity or even in the skin (56).

On the other hand, those lesions in which the presence of lesions in limited and non-bilateral areas have a different connotation and definition are today classified with the term Oral Lichenoid Lesions (OLL) where well-defined causal factors (dental materials, drugs, traumas) are of greater importance and led to an alteration of some self-antigens with respect to individual autoimmune

hyperreactivity (57). The OLLs, in turn, were then divided according to the causative agent into lichenoid contact lesions (OLCL, Oral Lichenoid Contact Lesions, especially amalgam-induced), drug-induced lesions (OLDR, Oral Lichenoid Drug Reaction) (58).

OLLs have also been detected in patients with other diseases with extraoral systemic involvement (HCV positive, lupus erythematosus, rheumatoid arthritis, Hashimoto's thyroiditis) (59). Characterized by fluctuating up abdanent in lesion morphology and in the variety of clinical symptoms, lichen is a chronic disease and if the cause is not identified, it rarely undergoes spontaneous remission.

There is no consensus to date on the

potential for malignant transformation of lichen, in fact, in 1997 the WHO included PLO among the potentially malignant diseases (60), but there are still no reliable studies able to quantify the extent of this risk and whether it is significantly greater than to the general population (61).

Clinical aspects: the cynical variability of a lichenoid lesion can take on different clinical aspects both for the distribution within the oral cavity and for the morphology of the lesion, and because it can take on a white-reticular color (striated, papular, plaque), or red-atrophic / erosive aspects (erosions, ulcers, blisters). The most identifying of the pathology are reticular and popular lesions, which manifest themselves with the appearance of intertwining and junction lines (Wickham's striae), while plaque lesions can be confused with leukoplastic lesions. On the other hand, reticular, popular or plaque lesions are asymptomatic, although they change appearance and, in some cases, extend to the entire oral cavity.

Atrophic-erosive lesions, on the other hand, are often symptomatic and the thinned epithelium seems to reflect the active form of the lichenoid reaction.

Histology: the presence of lattices as a clinical manifestation identifies lichenoid lesions in a particular way, which are strongly indicative for a diagnosis of OLP (62). Unfortunately, their identification was detected only in a low percentage of cases and therefore it is always recommended to perform a histological examination by biopsy of a representative area of the lesion.

To diagnose a "lichenoid reaction" the presence of a dense inflammatory infiltrate below the basal layer and composed mainly of lymphocytes, signs of interruption of the basal layer, presence of Civatte's bodies must be present (63). The immunohistochemical examination is essential to identify fibrinogen deposits and Civatte's bodies in the presence of erosive pictures and/or boils suspected of bullous diseases.

The absence of objective criteria for quantifying the probability of developing OLP cancer over time does not provide the possibility of a reliable initial diagnosis.

The problem occurs mainly in the OLL, when the lesions are isolated and the histological examination is sometimes unable to distinguish an "aggressive" lichen infiltrate typical of the OLL, from a "defensive" lymphocytic infiltrate present in some preneoplastic forms such as leuco / erythroplakias; in these cases a correct differential diagnosis is very difficult, and therefore, in case of neoplastic progression of the lesion, it is very difficult to establish whether the tumor has developed from a lichenoid lesion or from a leuco/erythroplakia (64). The impossibility of obtaining, therefore, an accurate distinction in the initial diagnosis has produced discordant results in the literature. Many authors consider OLL lesions to be at very low risk of malignant transformation, arguing that the lesions hesitated in tumors would actually be unrecognized forms of leuco/erythroplakia while others report overlapping risk rates with leuco/erythroplakia, with an increased risk especially for lichenoid dysplasia (DL).

1.4 Anatomical Sites and Pathological Features

1.4.1 Anatomical Sites

The anatomical distribution of the carcinoma can be varied, especially in consideration of the patient's age.

We will examine the major sites involved and the frequency of their involvement first in the elderly patient, then in the young patient, then in the age <45 years.

Aged patients

Taking into consideration elderly patients, the most affected site is the lower lip, with the main risk factor represented by exposure to sunlight, followed by the

tongue and the oral floor with a percentage of 30-40%, 25% respectively. and 20%.

Localization in the lower lip is very common, particularly between the ages of 50 and 80, mainly associated with exposure to sunlight that reaches the lower lip tangentially. It is mostly present in people who carry out particular outdoor professions, such as farmers, bricklayers or fishermen. Compared to the upper lip, the lower lip is affected with a 4: 1 ratio and represents 30-40% of oral carcinomas in non-young patients, often preceded by actinic keratosis, i.e. a precancerous growth that presents exposure to the sun, as well as advanced age, male gender, fair complexion and all immunosuppressive conditions as the main risk factors. It is estimated that more than 60% of squamous cell carcinomas derive from actinic keratoses, clarifying the importance of early diagnosis, i.e., in the precancerous phase, and early intervention (65).

The second most frequent site in the elderly patient is the Tongue. It manifests itself with a wide variety of clinical signs. Male sex is more affected, with a ratio of 9: 1, due to the habit of tobacco. In the elderly patient the incidence is mainly between 60 and 80 years. The posterior third of the lingual margin and lingual belly is the most frequent location, while it is rarer in the lingual dorsum.

The metastatic potential of carcinoma of the tongue is very early, particularly in the submandibular and deep cervical lymph nodes (66).

The third site of manifestation of oral carcinoma in the elderly patient is the oral floor. Often in this site the heteroplastic lesion is preceded by precancerous lesions such as leukoplakia or erythroplakia, therefore also here the importance of early diagnosis and of early intervention.

The most affected area is that corresponding to the exit of Wharton's duct, the excretory duct of the submandibular salivary gland, which opens to the side of the lingual frenum.

There may be frequent metastatic spread especially to the submandibular and jugular chain lymph nodes (67).

Young patients

The data emerging from the literature show that in patients aged <45 years the most frequently affected site of oral cavity cancer is the tongue. In fact, the

percentage of cases of tongue cancer is higher in the young patient than in the elderly patient; in contrast, the percentage of oral floor cancer cases is lower in the young patient than in the elderly patient (46) (68).

1.4.2 Histopathology

Squamous cell carcinoma accounts for over 90% of malignant tumors in the head and neck region, resulting from the mucosal epithelium. The diagnosis and sporadic management of these tumors can be very complex, despite the wide spread of the same. This is determined by the subjective nature of the diagnosis and classification over the wide range of variants, each with different diagnostic criteria, site predilection and biological perspectives. Although there are several variants with different histological and clinical characteristics which require different management and treatments, these differences can be very subtle. There may be crossovers in the appearance and cause of the injuries, and a rigid anatomical split is not always easy to apply.

The peculiar characteristics of the well differentiated forms highlight the presence of proliferating polygonal epithelial cells, arranged in nests, the keratinization can be more or less marked. It is possible to find "horny pearls", which are agglomerates of keratin inside the tumor mass, or intercellular bridges similar to the appearance of the spines, i.e. desmosomes, can be observed. The recognition of the less differentiated forms is more complex, in which the recognition of the cell type is difficult. Cell polymorphism and the number of mitoses increase, while the intercellular bridges disappear. In the poorly differentiated forms, it is no longer possible to recognize the epithelial origin of the cells, as these have lost all the characteristics that allow their recognition. In these cases, therefore, immunohistochemical evaluation becomes of fundamental importance to identify the expression of cytokeratin. However, it must be considered that frequently the different aspects analyzed can coexist within the same lesion, making it difficult to precisely define the degree of differentiation.

The presence of lymphocyte infiltrate has been associated with a more favorable prognosis as it could represent a response of the body's immune system towards

the cells that have undergone a neoplastic transformation, becoming "harmful" for the organism itself, which therefore recognizes them. as not-self.

In most cases the tumor has a marked local aggression, with the ability to invade the surrounding tissues, with advancement to nests or single cells. Spreading by contiguity, the neoplasm can affect bone structures, but the most typical spread is lymphatic, followed by blood (69) (70).

Verrucous carcinoma: verrucous carcinoma represents 2-12% of all oral carcinomas is represented by verrucous carcinoma which is considered a variant of low-grade squamous cell carcinoma due to its characteristics: well differentiated cells, with little or no atypia cellular, few corneous pearls and poor mitosis, with basically intact basement membrane (71).

Growth of the lesion is usually slow, as is invasion of surrounding structures. Regional and distant metastases are rare. Given these characteristics, it is believed that verrucous carcinoma has a relatively favorable prognosis.

The macroscopic appearance of the lesion is papillary-warty exophytic, with an irregular and rarely ulcerated surface, from which it takes its name. It frequently affects the buccal mucosa, tongue, lips, gums, and oral floor (72).

Other less frequent variants are represented by basaloid squamous carcinoma, adenosquamous carcinoma, papillary squamous carcinoma, lymphoepithelial carcinoma.

1.5 Diagnosis and staging

Over the years, early diagnosis has acquired a fundamental role, which leads to the possibility of early treatment and consequently to a treatment of early forms of staging. This allows the doctor, together with an accurate medical history, physical examination, and imaging diagnostics, to distinguish a neoplastic pathology from a more banal inflammatory pathology or to identify lesions in a precancerous phase. In fact, it is known that many neoplastic lesions of the oral cavity are preceded by precancerous lesions, the identification of which therefore becomes the key to giving the patient the best treatment and to ensure maximum survival.

1.5.1 Anamnesis

The diagnosis of a lesion of the oral cavity requires that the family anamnesis be investigated, the presence of a familiarity for neoplastic pathologies of the digestive system, but also of other parts of the organism; assess the patient's lifestyle, the presence of risk factors such as smoking or alcohol, exposure to ultraviolet rays, poor oral hygiene; the presence of precursors or precancerous lesions, diseases that have altered the patient's immune status or the presence of immunosuppression, liver disease; evaluation of the temporal and symptomatic aspect of a transformation of a persistent lesion: a colored spot or a scaly white spot that suddenly increases in size; gum ulceration or bleeding that does not tend to heal; impaired sensation such as hypoesthesia or paraesthesia; previous loss of symmetry in the face; difficulty swallowing or speaking.

Pain, often the presenting symptom of the disease for the patient, does not seem to be related to the size of the tumor, it is generally well localized and related to a function. A lingual or oral floor neoplasm could cause ear pain due to nerve connections with the tympanic cord (73).

1.5.2 Clinics and Physical examination

There are different clinical manifestations of the lesions, which change considering that it is an initial form rather than an advanced form, also from the symptomatologic point of view.

Early forms should be suspected especially when a cause is not identified or when there is no response to treatment of the presumed cause (two weeks of observation is more than enough to guide the diagnosis). Symptoms are often totally absent at the beginning or mild allergies may occur in contact with acidic, alcoholic, or hot substances. Periodic burning or mechanical discomfort may then appear during chewing, up to a more evident picture with dripping of blood, due to neoangiogenesis induced by the tumultuous growth of the lesion.

The growth of the lesion in the most advanced stages can lead to the infiltration of important structures, nerves, or muscles, creating a clinical picture characterized by functional limitations of speech, chewing or swallowing movements and by alterations in sensitivity. The onset of symptoms is very varied, it can range from the perception of changes in the tone of the voice, from

pain and difficulty during chewing and swallowing, to pain and stiffness of the jaw or sensation of a foreign body (74).

The fundamental focus of a correct diagnosis is the physical examination which consists of the inspection and palpation of the patient. It starts with a macroscopic evaluation of the lesion itself, taking into account color, appearance, location, margins, whether or not it is present with ulcerations. Palpation is then carried out, which allows the evaluation of the consistency, the conditions of the border and infiltration as well as the presence or absence of lymph node metastases. For the oral floor, bimanual palpation is required, performed by placing one hand in the submandibular region while the other evaluates the oral pelvis. The clinical appearance of the carcinoma can be distinguished in three modes of presentation: exophytic, endophytic and ulcerated.

The least aggressive and early diagnosed form is the exophytic form, which typically appears as an outgrowth detected on the healthy, multi-breasted, cauliflower mucosa, white or pinkish white, to which well differentiated forms generally correspond. The superficial proliferative form, of rare occurrence, goes into differential diagnosis with oral hairy leukoplakia.

The endophytic form has a nodular and an infiltrating variant. The nodular one appears as a hard lump underneath the healthy mucosa; the infiltrating one presents with hardening of the affected area associated with functional deficits such as inability to protrude or laterodeviation of the tongue, due to involvement of the chewing muscles.

The subtype handily present is the ulcerated form which appears as an ulceration with irregular margins, with sometimes raised hardened edges, with a hard consistency on palpation. The bottom is bloody, vegetating, sometimes covered with necrotic material. The combination of vegetative lesions with an ulcerated appearance results in ulcerative-vegetative lesions with a mixed appearance. The superficial erosive form presents itself as a red area with a mucous surface, velvety in appearance, irregular with white areas for which it goes into differential diagnosis with erythroplakia. (75) The ability to metastasize for oar carcinoma is mainly represented by metastases lymph nodes, while tissue

metastases are rarer and occur in very advanced stages of the disease (76). The frequency of lymph node involvement is related to the size and depth of the invasion, thickness, site, and differentiation. Generally, the first station to be affected is the submental or submandibular station (77). The evaluation of the lymph nodes is therefore a very important clinical aspect to consider, and this evaluation obviously includes size, consistency, mobility and shape. It is important to consider all aspects, because for example a regional lymph node enlargement does not necessarily indicate a metastatic spread and can only represent a non-specific reactive alteration.

1.5.3 Instrumental evaluation

Instrumental diagnosis is of fundamental importance in oral cancer, mainly in relation to staging as well as for the local extension of the disease. The occurrence of Metastases in OSCC per se, in the presence of an adequate interception of the patient, is considered a rare event, however there are sites where they can be found more frequently are in order of frequency the lung, liver and bone. However, the possibility of distant metastases is related to the involvement of the regional lymph nodes and in particular to their invasion and capsular rupture. There is still the possibility of distant metastases occurring in the absence of evidence of primary cancer in 28% of cases (78).

First level investigations are orthopantomography (OPG) and/or dental X-ray which are used to roughly assess the possible presence of bone infiltration of the neoplasm, as they provide a global view of the cervico-facial bone structures. the local stage of the disease, for the surgical planning, which for the lymph node invasion the execution of

CT and/or MRI of the facial massif and neck individually or in combination to ensure a complete skeletal and soft tissue evaluation. In cases where there are specific contraindications in performing MRI, obviously, only CT is used (79).

To perform a more complete staging of the disease, PET is increasingly associated with non-contrast CT which have taken on an important role in recent years. It can also be used in the evaluation of synchronous primary lesions (80).

1.5.4 Staging

The staging of patients with carcinoma of the oral cavity is delegated to the TNM system, which consists of three fundamental components, such as the size of the primary tumor (T), the presence of regional lymph node metastases (N) and distant metastases (M). The first edition of the TNM staging system dates to 1968 and the last and eighth edition wwasreleased in 2017 and went into effect in 2018 (81) (82).

The importance of this new edition is due to the introduction of a new parameter for the evaluation of T, the DOI, Depth of Invasion. A clinical TNM classification (cTNM) and a pathological TNM classification (pTNM) are distinguished. In the first, clinical data are examined, that is, data provided by objective examinations, imaging techniques, biopsies, and other specific examinations. In the second, the data already acquired are integrated with those derived from the histopathological evaluation of the lesion after the removal of the tumor, from the evaluation of the removed lymph nodes and from the microscopic examination of any metastases.

Primary tumor (T stage)

The introduction of the third dimension of the lesion in the eighth edition, has become fundamental in the staging role since it reflects the depth of infiltration. Primary tumors of the tongue and floor of the mouth, deeply infiltrating but clinically early stage, are known to have a higher risk of regional metastasis and death from disease than superficial cancers of the same stage T. Therefore, consideration may be required. a more aggressive therapeutic approach, including treatment of regional lymph nodes for patients with early stage but deeply infiltrating cancers. In contrast, lesions that are advanced in stage T but are relatively superficial have a better prognosis. The new AJCC staging system that addresses these issues in the new T staging for oral cancers will facilitate adequate treatment planning and prognosis assessment (83) (Figure 2).

	T – Oral cavity
T1	Tumour **2cm or less** in greatest dimension and **5 mm or less** depth of invasion (DOI).
T2	Tumour **2 cm** or less in greatest dimension and more than **5mm but no more than 10 mm DOI**, or Tumour **more than 2 cm but not more than 4 cm in greatest dimension and DOI no more than 10 mm.**
T3	Tumour more than **4 cm** in greatest dimension **or more than 10 mm DOI.**
T4	T4a (Lip) Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth or skin. T4a (Oral cavity) Tumour invades through the cortical bone of the mandible or maxillary sinus, or invades the skin of the face. T4b (Lip and Oral cavity) Tumour invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery.

Figure 2. © Brierley J, Gospodarowicz M, Wittekind C. International Union Against Cancer (UICC) TNM Classification of Malignant Tumours, 8th edition. Oxford, Wiley, 2017

Regional lymph nodes (N stage)

The introduction of the new lymph node staging system includes important prognostic parameters such as the location of the metastatic lymph node, as well as the presence of capsular penetration and extranodal extension (Figure 3).

	Oropharynx-p16 Negative, Hypopharynx, Larynx and Oral cavity Tumours
NX	Regional lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Metastasis in a **single ipsilateral lymph node, 3cm** or less in greatest dimension **without extranodal extension (ENE).**
N2	N2a Metastasis in a **single ipsilateral** lymph node more **than 3 cm but not more tan 6 cm in greatest dimension without ENE.** N2b Metastasis in **multiple ipsilateral** lymph nodes, **none more than 6 cm in greatest dimension, without ENE.** N2c Metastasis in **bilateral or contralateral** lymph nodes, **none more than 6cm in greatest dimension, without ENE.**
N3	N3a Metastasis in lymph node **more than 6 cm in greatest dimension without ENE.** N3b Metastasis in a single or multiple lymph **nodes with clinical ENE.**

Figure 3. © Brierley J, Gospodarowicz M, Wittekind C. International Union Against Cancer (UICC) TNM Classification of Malignant Tumours, 8th edition. Oxford, Wiley, 2017

Distant metastases (M stage)

Although there may be the absence of specific symptoms, it is always advisable to carry out a staging exam to exclude the possibility of the presence of distant disease which is always referred to as M0 or M1. In this scenario, the examination of choice is PET / CT which provides useful elements in advanced T or N stages (84).

1.5.5 Grading

In order to identify and study the microscopic characteristics of the heteroplastic tissue, a biopsy examination is always necessary, which can be excisional or incisional, the latter preferred in the case of suspected malignant and larger lesions.

In case you are faced with multiple or irregular lesions, it is particularly recommended to take multiple samples associated with portions of healthy tissue. This allows you to carefully evaluate type and histological grade. Sometimes in cases of lesions that are anatomically difficult to reach through a classic biopsy examination, the use of FNA-B needle biopsy (Fine Needle Aspiration Biopsy) is recommended (85).

In consideration of the histopathological characteristics, it is possible to classify the tissue in:

GX The differentiation grade can't be validated

G1 Well-differentiated carcinoma

G2 Moderately differentiated carcinoma

G3 Poorly differentiated carcinoma

G4 Undifferentiated carcinoma

1.6 Prognostic Indicators

In oral carcinoma the prognostic factors of the patients play a fundamental role, which are indicative of the biological aggressiveness of the neoplasm and therefore of the possible local evolution. In particular, OSCC, also in relation to the increase in average life, tends to affect subjects between the sixth and eighth decade of life. The question was therefore whether the age factor was an unfavourable prognostic factor or not. The results emerging from the literature

are controversial, Douglas R. Farquhar et al. studied cases of tongue cancer in the young and elderly population, analysing 397 cases in total, of which 117 (29%) with less than 45 years, and no differences in terms of survival were found (86).

There are other data in the literature that show how age can actually be a prognostic factor. Jeon JH et al. conducted a retrospective study of 117 cases of tongue cancer between 2001 and 2011, broken down by age if over or under 40 years. There was a significant difference in absolute and relative survival and distant metastasis-free survival: 5-year absolute survival was 70% in the population over 40 and 42% in the population under 40. relative was 73 and 40%, respectively, and distant metastasis-free survival was 97% and 62%, respectively, demonstrating that age is an important risk factor for long-distance metastasis (87).

1.6.1 Tumor Stage and Degree of differentiation

The stage of the disease is a predictor of prognosis. the AJCC has published data that the 5-year relative survival for oral squamous cell carcinoma by stage is as follows: stage I, 65 to 70%; stage II, from 50 to 55%; stage III, from 38 to 44%; and stage IV, from 25 to 29% (88). This validates the usefulness of the AJCC staging system as a predictor of prognosis and survival. Depending on the degree of differentiation, survival ranges from 93% (grade 1) to 70.9% (grade 3 and 4). The same is true for other sites, such as language, where survival varies from 67.4% to 36.4% (local and regional phase) and from 65.2% (class 1) to 41.1% (classes 3 and 4) (89).

1.6.2 Bone Invasion

Particular attention should be paid to the occurrence of bone invasion. During the publications of the AJCC Cancer Staging Manual the relationship between bone invasion and regional metastases has been clarified. Minimal cortical invasion is not insufficient to classify a tumor as a T4 lesion, but frank bone invasion is important from a prognostic point of view and univariate analysis has shown a negative impact on survival. (91) However, bone invasion was not a consistent independent predictor of survival on multivariate analysis. (92)

Although bone invasion may have prognostic significance, this association remains poorly defined.

1.7 Treatment

Tumors of the oral cavity basically provide for two types of treatment, palliative, and curative. The prognostic and performance status factors of the patient condition the type of treatment to which patients with cancer are subjected. Advanced stages of the disease argue for a reduction in the probability of success of the proposed treatments. In early states of the disease, the treatment to be preferred as a first choice is always the surgical one. Thanks also to the development of new surgical and reconstructive techniques, the impact on the patient's social life has significantly improved over the years (93).

Chemotherapy

The use of chemotherapy is known to be chosen for a variety of purposes in both curative and palliative treatment to improve survival, control the spread of the disease by limiting the incidence of metastases. There is the possibility of using it as a neoadjuvant therapy, integrated with radiotherapy or as an adjuvant therapy after locoregional therapy.

The choice of neoadjuvant treatment is based on the administration of drugs before locoregional therapies, and the protocol used provides for the combination of cisplatin and 5-fluorouracil. It has been described the possibility of use in patients with advanced disease, multiple lymph node metastases, extracapsular lymph node invasion, endovascular or neural neoplastic infiltration, after locoregional treatment local chemotherapies can be administered in an attempt to improve local control and contrast the systemic spread of the disease. (94).

In order to be used in an attempt to obtain a reciprocal enhancement of the therapeutic effects of the individual methods, chemotherapy integrated with radiotherapy administered in symmetrical or alternative form, is one of the possible therapeutic choices proposed for patients with neoplasia. They are often preferred for the treatment of advanced disease, with the intent of preserving organ and function, proving effective in controlling local disease, unfortunately burdened by a high level of toxicity (95).

New therapeutic frontiers have been proposed to patients thanks to the introduction of a new treatment plan for oral cancer, namely the inhibition of the signal transduction cascade of the epidermal growth factor receptor (EGFR) through the use of a monoclonal antibody anti-EGFR, Cetuximab.

Radiotherapy

Radiotherapy, which unlike chemotherapy is rarely used as a neoadjuvant approach, can be a therapeutic choice associated with surgery and/or chemotherapy, but it can also be the only therapeutic approach employed. On the other hand, it plays a fundamental role as an "adjuvant", i.e. postoperative, therapy in order to induce the necrosis of any residual neoplastic cells to avoid and prevent tumor recurrence. Furthermore, if the patient has a very advanced stage of cancer, "palliative" radiotherapy is used to improve the quality of life, to reduce symptoms of pain, compression and/or infiltration (96).

The introduction of intensity modulated radiotherapy (IMRT), has brought great benefit to clinical practice for the selectivity and precision of the treatment, resulting in a significant improvement by adapting the gradients to the anatomical characteristics of the target areas, given the local anatomical complexity and the presence of many critical and radiosensitive organs (97).

Brachytherapy is sometimes used as an alternative to traditional chemotherapy, which, as shown in recent studies, has excellent results in local control of the disease over 80% in the early stages (T1-T2-N0). In addition, resulting in fewer side effects also allows the possibility of subsequently performing conventional radiotherapy in the case of a second primary tumor (98).

Surgery

The possibility of performing surgical treatment remains to this day the best therapeutic choice to be offered to patients suffering from OSCC. The use of surgical and reconstructive techniques guarantees a more careful evaluation of the lesion, margins and histopathological characteristics (99). This information is also useful for patient management after treatment.

Where possible, in the presence of small lesions, the surgical approach involves the use of trans-oral techniques. If we are faced with more extensive lesions, the use of more invasive techniques must be envisaged (100).

Often in consideration of the stage of the disease and the presence or absence of local-regional lymph node metastases, an emptying of the later cervical lymph nodes is associated also with prophylactic purposes, possibly operating in T and N monoblocs. Locoregional lymph nodes are an important prognostic factor, which significantly reduces patient survival. About 60% of patients with early-stage oral cancer have a cN0 condition, which is clinical negativity of the lymph nodes in the neck. About 20-30% of patients, after elective neck dissection (END), show the presence of microscopically evident metastases on histological examination. This underlines the importance of neck treatment, particularly in places where lymph node spread is particularly frequent, where such treatment would also be indicated in cases of "early stage" cancers (101).

1.8 microRNAs

microRNAs (miRNAs) are small single-stranded non-coding RNA molecules of 18-25 nucleotides in length. miRNAs are involved in the post-transcriptional regulation of gene expression through the inhibition of mRNA translation mediated by the recognition of specific mRNAs region (3'-UTR and 5'-UTR). In particular, miRNAs are able to block the translation of mRNA into protein promoting the complete degradation of targeted mRNA or temporarily inhibiting the access of mRNA to the ribosome. Specifically, if miRNAs and targeted mRNAs have a 100% complementarity the mRNA is totally degraded by the miRNA-RISC complex, while if miRNAs and targeted mRNAs are partially complementary, the miRNA-RISC complex only inhibits mRNA translation without inducing the degradation of the target.

At present, the functions of many miRNAs have not completely clarified yet; however, some miRNAs have been recognized as mediators of some pathological or physiological processes. Furthermore, it has been shown that their regulatory mechanism is altered in some human diseases in which miRNAs play an important role in tumorigenesis mechanisms. In complex organisms, miRNAs regulate a wide range of biological processes, including development, cell differentiation, proliferation, cell metabolism and apoptosis (102).

To date, there are international databases in which miRNAs characteristics and functions are recorded. Among these, the most important is miRBase, which

provides information on the gene regions and the nucleotide composition of each miRNA.

1.8.1 Biogenesis of microRNAs

The biogenesis of miRNAs begins with precursors miRNA, which are found mostly in the intergene and intronic regions of DNA but less commonly in the exons. Intergenic miRNAs are transcribed by RNA polymerase II, generating a primary miRNA (pri-miRNA) molecule, which is processed into a precursor miRNA (pre-miRNA) by the microprocessor complex comprised of DGCR8 and Drosha and a class III endoribonuclease.

This protein complex removes the 3'-5' ends of the first-miRNA molecule allowing to obtain a double-stranded pre-RNA molecule in stem-loop conformation. Pre-miRNAs are exported to the cytoplasm in a nucleocytoplasmic transporter containing Exportin 5 and Ran-GTP proteins. Within the cytoplasm, pre-miRNAs undergo a further modification by the Dicer-TRBP protein complex, another third-class endoribonuclease that is able to recognize the double-stranded miRNA and make cuts in specific positions to transform the molecule of pre-miRNA in 2 mature single-stranded miRNAs of approximately 22 bases. Subsequently, these molecules interact specifically with Argonaut (Ago) proteins, which stabilize the single-strand miRNA that will become functional, while the opposite filament is degraded. The mature single-stranded miRNAs are then loaded into the miRISC complex (MiRNA-associated multiprotein RNA-induced silencing complex) that favors the interaction between miRNA and targeted mRNA. As mentioned before, the miRNAs can have two different ways of acting depending on the complementarity that exists between them and their targets. Indeed, in the case of perfect complementarity there is the degradation of the target mRNA molecule, while if there is an imperfect complementarity (not 100%), the translation process is blocked (103).

1.8.2 Regulation of microRNA expression

The regulation of miRNAs expression is mediated by different factors. miRNA processing can be regulated in multiple steps and leads to the up-regulation or down-regulation of these molecules in specific physio-pathological conditions

(104). Regulatory proteins that affect miRNA processing, acquired changes in miRNA transcript, and changes in nuclear export efficiency can cause altered levels of miRNA. In addition to these regulatory mechanisms, single nucleotide polymorphisms (SNPs) or DNA methylation can also have a pronounced effect on the efficiency of the miRNA processing machinery. Regulation occurs both at the transcriptional level (changes in gene expression and promoter hypermethylation) and at the post-transcriptional level (changes in miRNA processing) (105).

Furthermore, various physiological and pathological stimuli, such as steroid hormones or stress, can influence miRNA expression. Several studies have demonstrated that estrogen can affect miRNA expression in breast cancer cells (106). The expression profile of microRNAs also changes in tumors under hypoxic conditions. It has been shown in many studies that factors such as hypoxia, variety of cell types such as fibroblasts and cancer-associated macrophages can further influence microRNA expression (107).

1.8.3 Clinical role of microRNAs

Although miRNAs play important roles in healthy individuals, they have also been implicated in a wide range of diseases: cardiovascular, neurological, diabetes and obesity. Consequently, miRNAs are being studied in the clinical setting as diagnostic and prognostic biomarkers (108). Altered expression profiles of miRNAs have been observed in specific tumors, demonstrating how these molecules may also be involved in cancer development. Experimental approaches have indicated that some miRNAs act as tumor suppressors and others as oncogenes (109); for this reason, they have important roles in cancer development, disease progression and prognosis.

In particular, determining the expression profiles of miRNAs is useful in many fields:

- Prognostic evaluation of the patient;
- To predict the effectiveness of the treatment and follow it over time the response to drug therapy;
- To study the patient's susceptibility to cancer and metastases.

These properties of miRNAs have been already validated for lung cancer, in which miRNAs allow to distinguish tumor cells from normal, as well as being correlated with the patient's prognosis (110). Furthermore, miRNA expression patterns have been shown to have relevance for the biological and clinical behavior of human B-cell chronic lymphocytic leukemia and solid tumors, including oral cancer (111). Certainly, the field in which miRNAs are having the most success is in the control of therapy, where they are used as predictors of response. Therefore, different chemotherapy drugs depending on their mechanism of action could have significant effects on the expression profile of miRNAs.

Recently several studies have started to test the therapeutic potential of miRNAs. Some researchers have used miRNAs as target therapy, with antagomir, chemically modified oligonucleotides that competitively bind miRNAs, inhibiting their expression (112). An example of the application of this therapy has been studied in hepatic metabolic diseases, the target of which is miR-122. In an in vivo study on obese and normal mice this method was used to inhibit, the hepatic expression of miR-122. The results obtained showed an evident decrease in plasma cholesterol levels, both in obese and normal mice. This study demonstrates the possibility of using antagomir as an innovative treatment, given their characteristics of stability, specificity and reduced toxicity (113). However, the use of antagomir as target therapy still has limitations today. Their mechanisms of action should be further studied and understood, in order to identify more precisely all the gene targets and their respective and suitable drug therapy (114).

1.8.4 Cancer and microRNAs

Several studies have analyzed microarray or NGS data of miRNA expression carried out in oral cancer samples. However, conflicting results about the miRNAs involved in the development and progression of oral cancer were generated mainly due to the use of different platforms of potential bias related to independent studies. Below are reported some studies on miRNAs and oral cancer.

Xiao et al. have identified 24 over-expressed miRNAs and nine down-regulated miRNAs by analyzing seven malignant oral lesions (OL) and 20 pre-cancerous lesions (115). Other research groups have shown that only miR-31 is overexpressed in both buccal gum SCC and OL, using tissue samples (116). However, the most consistent article describing a number of miRNAs associated with pre-cancerous oral lesions is the study of Cervigne et al. (2009) which examined the expression levels of miRNAs in 29 OL that progressed in OSCC and in 7 normal tissues. In this study the authors identified a high number of significantly altered miRNAs in the tumor compared to precancerous and healthy tissue. In particular, miR-21, miR-181b and miR-345 showed increased expression during progression to OSCC (117).

In addition, the study highlighted how the increased expression of miR-345, whose perfect functional mechanism is not yet well understood, seems to induce down-regulation of the BAG3 gene, an anti-apoptotic molecule involved in the development of OSCC.

In a study conducted by Manikandan M. et al. in an exploratory cohort (n = 29) and a validation cohort (n = 61) of primary OSCC tissue samples, the authors identified a set of miRNAs let-7a, let-7d, let7f, miR-16, miR-29b, miR-142-3p, miR-144, miR-203, potentially involved in the development and progression of oral cancer as these miRNAs actively altered the expression of different genes belonging to signal transduction and cell cycle regulation pathways (118).

2. AIM OF THE STUDY

As described in the Introduction section, oral cancer is often diagnosed late when the tumor is in an advanced stage. The reason behind the late diagnosis of oral cancer is related to the lack of effective biomarkers for oral cancer. Although several studies have tried to identify novel effective biomarkers for oral cancer, no factors have been identified for the reliable diagnosis of this tumor. Therefore, the aim of the study was to identify novel biomarkers for the effective diagnosis of oral cancer. In this context, several studies have demonstrated the high diagnostic and prognostic value of the alteration of the expression levels of microRNAs (miRNAs) in different pathologies, including tumors, therefore, miRNAs can represent promising biomarkers for patients with oral cancer, due to their stability and non-invasive detectability in blood and saliva samples.

On these bases, the purpose of the present study was to identify miRNAs potentially involved in the development and progression of oral tumors through the analysis of miRNA expression expression bioinformatic data contained in the GEO DataSets and The Cancer Genome Atlas Head and Neck Cancer databases. Differential analyses were performed between the expression levels of miRNA observed in the tumor and normal samples. In addition, several bioinformatics tools were used to establish the functional roles of these miRNAs, in particular with regard to their involvement in neoplastic transformation and in the development of oral cancer.

Four of the computationally identified miRNAs potentially involved in oral cancer development and progression were subsequently validated in liquid biopsy samples, both serum and saliva samples, obtained from a pilot cohort oral cancer patients, OLP patients and normal individuals as controls. For this purpose, the high-sensitive droplet digital PCR amplification system was used.

The miRNAs thus validated were further analyzed through computational approaches to establish also their prognostic significance and functional roles in oral cancer.

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3. MATERIALS AND METHODS

3.1 Selection of microRNA expression profiling dataset from GEO DataSets and The Cancer Genome Atlas Databases

The “The Cancer Genome Atlas Head and Neck Cancer (TCGA HNSC)” and the “Gene Expression Omnibus (GEO) DataSets” (www.ncbi.nlm.nih.gov) databases were analyzed to select the microarray miRNA expression datasets contained the expression levels of miRNAs in both tumor and normal samples of oral cancer patients. The selection of these datasets is useful for the computational identification of miRNAs potentially associated with oral cancer development and progression. As regards the TCGA HNSC datasets, the miRNA mature strand expression RNAseq IlluminaHiseq dataset was selected while for the GEO DataSets database all oral cancer miRNA expression datasets were identified by using the following search terms: "((oral cancer) AND 'non coding rna profiling by array' [DataSet Type]) AND 'Homo sapiens' [porgn: __ txid9606].

After the selection of datasets, differential analyses between the expression levels of miRNAs in oral cancer patients compared to healthy controls were performed. For the TCGA HNSC miRNA expression dataset, the data matrix of miRNA was downloaded using the XENA Browser platform. The differential analyses of these data were performed manually, and the fold change values were expressed as log2 expression levels of miRNAs in cases and controls. For the datasets selected from GEO DataSets database, differential analyses were performed using the publicly available tool GEO2R. The values of differential expression were expressed as base 2 logarithm of the fold change (log2FC). Only differentially expressed miRNAs with a p-value $p < 0.01$ were considered as potentially involved in oral cancer development.

3.2. Patients and samples

To confirm and validate the diagnostic or prognostic potential of the selected miRNAs, a pilot cohort of oral cancer patients, oral lichen planus patients and healthy individuals were enrolled. From both patients and controls liquid biopsy

samples consisting of blood and saliva samples were collected. More in detail, ten healthy donors, ten oral cancer patients and 14 oral lichen planus patients were enrolled in this study in order to validate the prognostic value of four miRNAs, hsa-miR-196a-5p, hsa-miR-133a-3p, hsa-miR-375-3p and hsa-miR-503-5p. For each individual, two peripheral blood draws were collected in order to separate serum (tube with separating gel) and plasma, buffy coat and red cells (tube with K3 EDTA) for future analysis. For each individual, 3 mL of saliva were also collected as a further liquid biopsy sample where to investigate the expression levels of the selected miRNAs. Both saliva and blood samples were centrifuged at 2,000 g for 10 minutes at room temperature in order to obtain different aliquots of serum, plasma, buffy coat and red cells. The samples were then stored at -80°C until their use.

The sociodemographic and clinical features of the subjects included in this study are reported in Table 1.

Table 1. Clinical pathological characteristics of patients and controls.

	Normal Individuals (N. 10)		OLP Patients (N. 14)		Cancer Patients (N. 10)	
	N.	%	N.	%	N.	%
Sex						
Male	6	60	6	42.86	6	60
Female	4	40	8	57.14	4	40
Age						
<45	0	0	2	14.29	0	0
45-59	6	60	7	50.00	3	30
>60	4	40	5	35.71	7	70
Smoke						
Yes	3	30	5	35.71	4	40
No	5	50	7	50.00	4	40
Ex smoker	2	20	2	14.29	2	20
Alchol						
Yes	1	10	3	21.43	3	30
No	9	90	11	78.57	7	70
Family History						
Yes	3	30	5	35.71	6	70
No	7	70	9	64.29	4	30

3.3 microRNA Extraction and Reverse Transcription

For the evaluation of miRNA expression levels in liquid biopsy samples, miRNAs were extracted by using an innovative protocol for the effective isolation and amplification of low expressed miRNAs from different biological

fluids including saliva, serum, plasma, CSF and urine developed by the research group of the Experimental Oncology Laboratory of the University of Catania.
Through the following protocol, the circulating miRNAs were extracted from saliva and serum samples obtained from the individuals enrolled in the study.
First, the miRNeasy Serum/Plasma kit protocol was adapted for the effective extraction of miRNAs both from serum and saliva.
Briefly, serum and saliva samples were centrifuged at 2,000 g × 10 min at room temperature to pellet down debris and protein aggregates. Then, 200 μL of serum or saliva was subsequently extracted using the miRNeasy Serum/Plasma kit (Cat. No. 217184, Qiagen, Hilden, Germany). Before the extraction, the UniSP4 artificial exogenous control was added to normalize the absolute quantification of extracted miRNAs (Cat. No 219610, Qiagen, Hilden, Germany). To optimize miRNAs extraction and purification molecular biology grade chloroform was used (Serva cat. n. 39553.01). After RNA extraction, miRNAs were selectively reverse transcribed into cDNA using the miRCURY LNA RT Kit (Qiagen – Cat. N. 339340). The miRCURY LNA RT technology ensures the efficient polyadenylation of miRNAs and the subsequent reverse transcription into cDNA in a single reaction step.

3.4 ddPCR for miRNA Expression Levels

For the analysis of miRNA expression levels in saliva and serum samples, a custom Qiagen-Bio-Rad hybrid protocol was adopted using specific primers specific for the four selected miRNAs as well as for the exogenous normalizer Unisp4 spike-in control (miRCURY LNA miRNA PCR Assays x200, Qiagen - Cat. No. 339306).
In particular, ddPCR was used to better evaluate the expression levels of miRNAs in samples with a low amount of miRNAs as liquid biopsy samples.
Also the amplification protocol reported below was developed by the Experimental Oncology Laboratory of the University of Catania in order to evaluate the expression levels of the two predicted down-regulated miRNAs hsa-miR-133a-3p and hsa-miR-375-3p and the predicted up-regulated miRNAs hsa-miR-503-5p and hsa-miR-196a-5p by using droplet digital PCR (ddPCR). The

same ddPCR protocol was adopted for the analysis of miRNAs obtained from serum and saliva samples.

Before, ddPCR amplification, cDNA samples were diluted 1:10 in sterile H_2O. Before use, the cDNA and all the ddPCR reagents were thawed on ice. The ddPCR reaction mix for the generation of the droplets was obtained as described in detail below:

- 11 μL of 2x QX200TM ddPCRTM EvaGreen® Supermix (Cat. N. 1864034 – Bio-Rad, Hercules, California, USA);
- 1.1 μL of miRCURY LNA miRNA PCR Assays (x200), i.e. specific primers for the three selected miRNAs and for the Unisp4 spike-in control (Qiagen - Cat. N. 339306);
- 6.9 μL of RNase and DNase free-water;
- 3 μL of cDNA (diluted from pure to 1:10) in order to obtain a final volume of 22 μL.

Subsequently, 20 μL of the ddPCR reaction mix were used to generate 20,000 droplets with the QX200 droplet generator (Bio-Rad, Hercules, California, USA) through specific cartridges with wells for amplification mixture and droplet generation oil. Through the vacuum operated by the droplet generator an oil-water emulsion was obtained in the form of thousands nano-droplets each containing a single copy of target cDNA and the components of the reaction mixture. After droplet generation, the droplets were transferred into a 96-well plate sealed with an aluminum foil and amplified using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, California, USA) according to the following thermal conditions:

- 5 minutes at 95°C for Taq polymerase activation;
- 40 cycles of amplification consisting of:
- first step of denaturation at 95°C for 30 seconds;
- second step of annealing and amplification at 56°C for 1 minute;
- 5 minutes at 4°C and subsequently 10 minutes at 98°C for droplet signal stabilization;
- infinite hold at 4°C.

A ramp rate of 2°C/s was used between each step/cycle of the amplification. After amplification, negative and positive signals were read in the QX200 Droplet Reader (Bio-Rad, Hercules, California, USA) using a capillary tube where every single droplet flow and a laser excitation source plus a detector to collect the fluorescent signals of positive droplets. All experiments were performed in triplicate.

Finally, the results were analyzed by using the QuantaSoft software, which allows the statistical analysis of the fluorescent data by applying specific correction factors, including the Poisson distribution, used for the evaluation of the copies of miRNAs contained in 1 µL of ddPCR reaction (Figure 4).

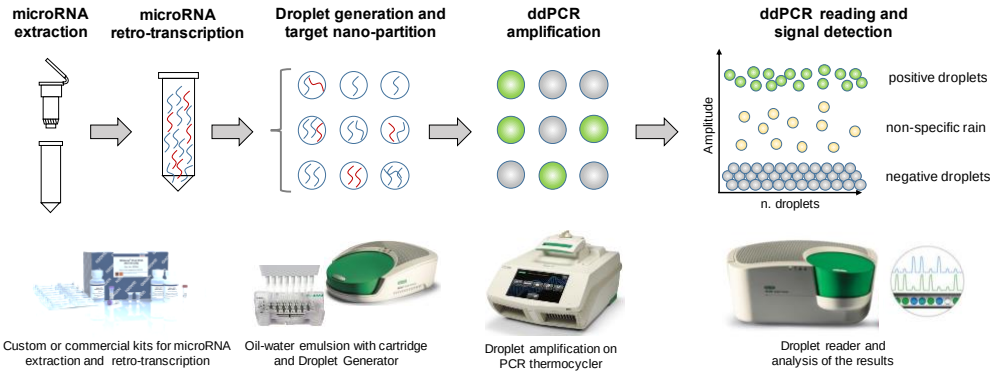

Figure 4. Analysis of miRNAs through Droplet Digital PCR (ddPCR).

3.5 miRNA-Gene Interaction of the Validated miRNAs

To evaluate the association existing between miRNAs dysregulation and oral cancer development, the interaction levels between the miRNAs validated by ddPCR with the main genes altered in oral cancer were established. First the most altered and mutated genes in oral cancer were identified by using the catalog of somatic mutation in cancer (COSMIC). Then, the microRNA Data Integration Portal bioinformatics tool (Version 4.1.11.1, Database version 4.1.0.3) (http://ophid.utoronto.ca/mirDIP) was used to evaluate the interaction levels existing between the selected miRNAs and the altered COSMIC genes.

To further confirm the pathogenetic role of the selected miRNAs in oral cancer, a pathway prediction analysis was performed by using the computational tool DIANA-miRPath V.3. Through DIANA-miRPath it was possible to identify the molecular pathways and related genes altered by the selected miRNAs. In

particular, this analysis was performed by analyzing the miRNA interactions obtained from the database DIANA-TarBase v7.0. The detected interactions, whether validated or predicted, can be then combined with fusion algorithms and meta-analysis thanks to the DIANA-mirPath tool.

To further establish the pathogenetic involvement of the validated miRNAs in oral cancer, other computational analyses using prediction software were performed. First, the genes targeted in common by two or three selected were identified using the miRTargetLink Human bioinformatics tool (https://ccb-web.cs.uni-saarland.de/mirtargetlink/multinet.php). In this way, the genes targeted by hsa-miR-133a-3p, hsa-miR-375-3p and hsa-miR-503-5p were identified according to the interaction data contained in the latest version of miRTarBase. Subsequently, the interaction network existing between the miRNA-targeted genes was established by using STRING v11.0 (119). In addition, the molecular pathways and biological and molecular functions of the miRNA-targeted genes were evaluated using the GO PANTHER v15.0 software (120)

To evaluate the epigenetic modulation operated by the selected miRNAs in the miRNA-targeted gene identified, the GEPIA portal was used to determine whether the genes targeted by hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p are differentially expressed in oral cancer and normal controls (121). Finally, the prognostic role of hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p was evaluated by analyzing the miRNA expression data and the clinical-pathological features contained in the TCGA HNSC database obtained using the UCSC Xena Browser. Tumor stage and lymph node status were taken into account as clinical parameters (122).

3.6 Statistical analysis

As regards the computational analyses performed to identify a set of miRNAs potentially involved in the development and progression of colorectal cancer, all the expression data of miRNAs obtained from GEO Datasets were already normalized by GEO2R software, therefore, no additional normalization procedures were applied to data obtained from all datasets included in this study. The statistical significance of the differentially expressed miRNAs was also

calculated by GEO2R. As regards the data obtained from the TCGA HNSC database, the fold-change values were calculated through specific formula on Excel, while the statistical significance of data was calculated by using Mann-Whitney non-parametric test on GraphPad Prism software.

For the data generated by ddPCR, the absolute quantification of miRNA expression levels was automatically performed by the QuantaSoft software (Bio-Rad). Normality tests were used to assess the distribution of miRNA n. copies/μL and for the data obtained from TCGA Head and Neck Cancer database. Mann-Whitney non-parametric test was used to evaluate the statistical difference between two groups of samples; while Kruskal-Wallis non-parametric test (and the post-hoc Dunn multiple comparison test) was used to assess the statistical differences existing among the expression levels of OLP patients, oral cancer patients and healthy controls. The sensitivity and specificity of the three selected miRNAs were assessed by performing the receiver operating characteristics curve (ROC). Kruskal-Wallis test (and the post-hoc Dunn multiple comparison test) and the one-way ANOVA test (and the post-hoc Dunnett multiple comparison test) were used to assess the statistical differences between the expression levels hsa-miR-503-5p, hsa-miR-375-3p and hsa-miR-133a-3p according to the tumor stage and lymph node status of TCGA HNSC patients. All statistical analyses were performed using the GraphPad Prism v.8 program.

4. RESULTS

4.1 Computational Identification of miRNAs Involved in Oral Cancer Development

By analyzing the miRNA expression data contained in both the GEO DataSets and TCGA HNSC databases it was possible to identify a set of miRNAs dysregulated in oral cancer compared to healthy controls. More in detail, three datasets were obtained, one from the TCGA HNSC database, i.e. the "miRNA mature strand expression RNAseq IlluminaHiseq" dataset, and two obtained from the GEO DataSets database, i.e. GSE45238 and GSE31277.

The differential analyses performed allowed the identification of three independent lists of dysregulated miRNAs in oral cancer. In detail, the analysis of the TCGA HNSC miRNA expression data revealed a list of 514 significantly dysregulated miRNAs ($p<0.01$) in oral cancer patients compared to normal controls. Of these the top 25 most over-expressed miRNAs and the top 25 most down-regulated miRNAs were selected as candidate biomarkers for oral cancer (Table 2).

Table 2. Differentially expressed miRNAs in the two GEO datasets analyzed

miRNA ID	GSE45238		GSE31277	
	Fold Change	p_Value	Fold Change	p_Value
Up-regulated miRNAs				
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 miRNAs				
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-375-3p	-16.589	1.95E-17	-3.198	5.12E-04

As regards the analysis of the two miRNAs expression datasets obtained from GEO DataSets database, a list of 28 miRNAs differentially expressed ($p<0.01$) in oral cancer samples of both datasets was identified. Of these miRNAs, 12 were up-regulated and 16 were down-regulated (Table 3).

Table 3. Up-regulated and down-regulated miRNAs in tumor samples compared to the healthy controls.

miRNA ID	GSE45238		GSE31277	
	Fold Change	p-value*	Fold Change	p-value*
Up-regulated miRNAs				
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 miRNAs				
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 obtained by using the GEO2R software by performing Student's t-test.

By comparing the results obtained from the TCGA HNSC and GEO DataSets a set of 11 microRNAs significantly dysregulated in oral cancer patients compared to healthy controls was identified. Of these miRNAs, seven were down-regulated and four were up-regulated (Table 4).

Table 4. List of oral cancer dysregulated miRNAs after GEO DataSets and TCGA HNSC data merging

miRNA name	GEO DataSets				TCGA HNSC	
	GSE45238		GSE31277			
	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	5.01	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	-1.62	1.71E-06	-3.51	7.79E-14
hsa-miR-411-5p	-5.574	3.25E-16	-2.542	6.18E-03	-4.16	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 were already calculated by GEO2R software; **p-values were calculated by applying Student's t-test

Of these 11 dysregulated miRNAs, the two up-regulated miRNAs hsa-miR-196a-5p and hsa-miR-503-5p and the two down-regulated miRNAs hsa-miR-133a-3p and hsa-miR-375-3p were among the most significantly and highly altered in oral cancer, therefore, these four miRNAs were selected for further validation studies.

4.2 Expression Levels of the Four Selected miRNAs in Liquid Biopsy Samples of Oral Cancer Patients, OLP Patients and Normal Controls

In order to validate the potential diagnostic value of hsa-miR-196a-5p, hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p, the expression levels of these miRNAs were evaluated on saliva and serum samples obtained from a case series of 34 healthy controls, OLP patients and oral cancer patients. The high-sensitive ddPCR amplification system was used to detect also slight variation in the expression levels of the selected miRNAs.

Of note, no amplification signals were obtained for hsa-miR-196a-5p analyzing both serum and saliva samples, therefore, this miRNA was excluded from the analysis.

The ddPCR analyses performed on serum samples revealed a significant increment of hsa-miR-503-5p expression levels in OLP patients compared to control samples ($p=0.0185$; Figure 5A) while no significant data were observed for hsa-miR-133a-3p (Figure 5B) and hsa-miR-375-3p (Figure 5C) levels.

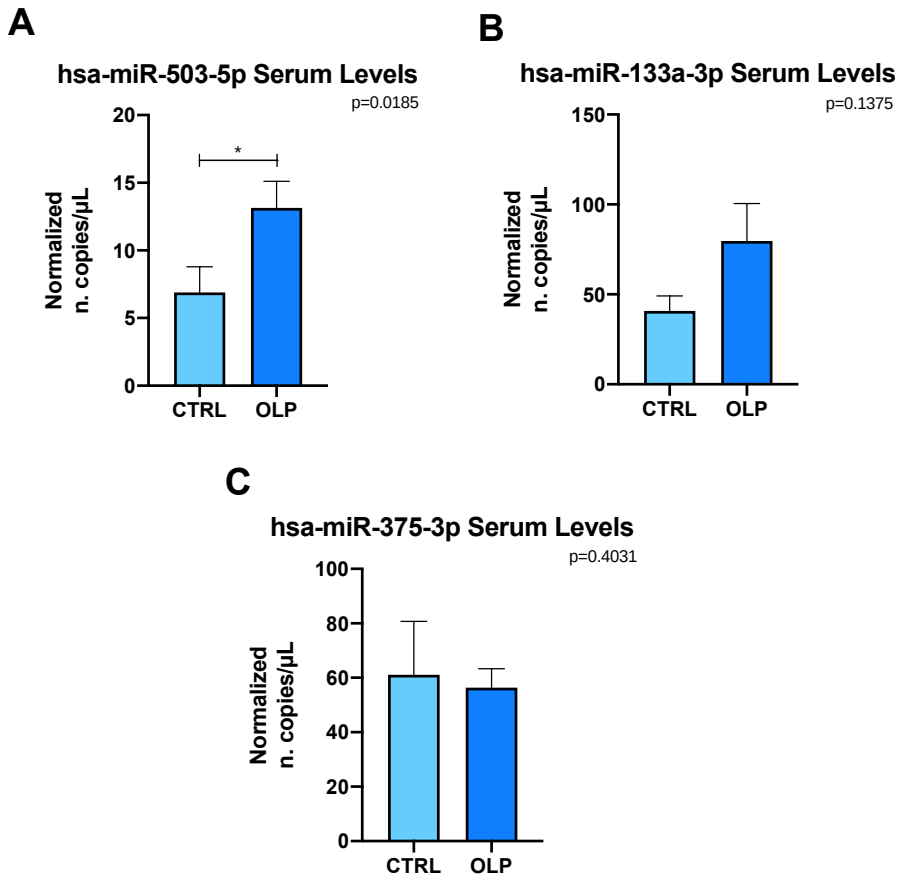

Figure 5. Expression levels of the three selected miRNAs, hsa-miR-503-5p **(A)**, hsa-miR-133a-3p **(B)** and hsa-miR-375-3p **(C)**, in oral lichen planus patients and normal controls. Student t-test: * $p<0.05$.

Similarly, the same analyses performed on saliva samples revealed strong statistical differences for the predicted up-regulated hsa-miR-503-5p whose expression levels were strongly increased in OLP saliva samples ($p<0.0001$; Figure 6A). In the case of saliva samples, also the expression levels of hsa-miR-133a-3p were significantly reduced in OLP patients compared to controls ($p=0.0155$; Figure 6B) further confirming the bioinformatics results previously obtained. Although no significant results were obtained for hsa-miR-375-3p, a slight decrement was observed in OLP saliva samples (Figure 6C).

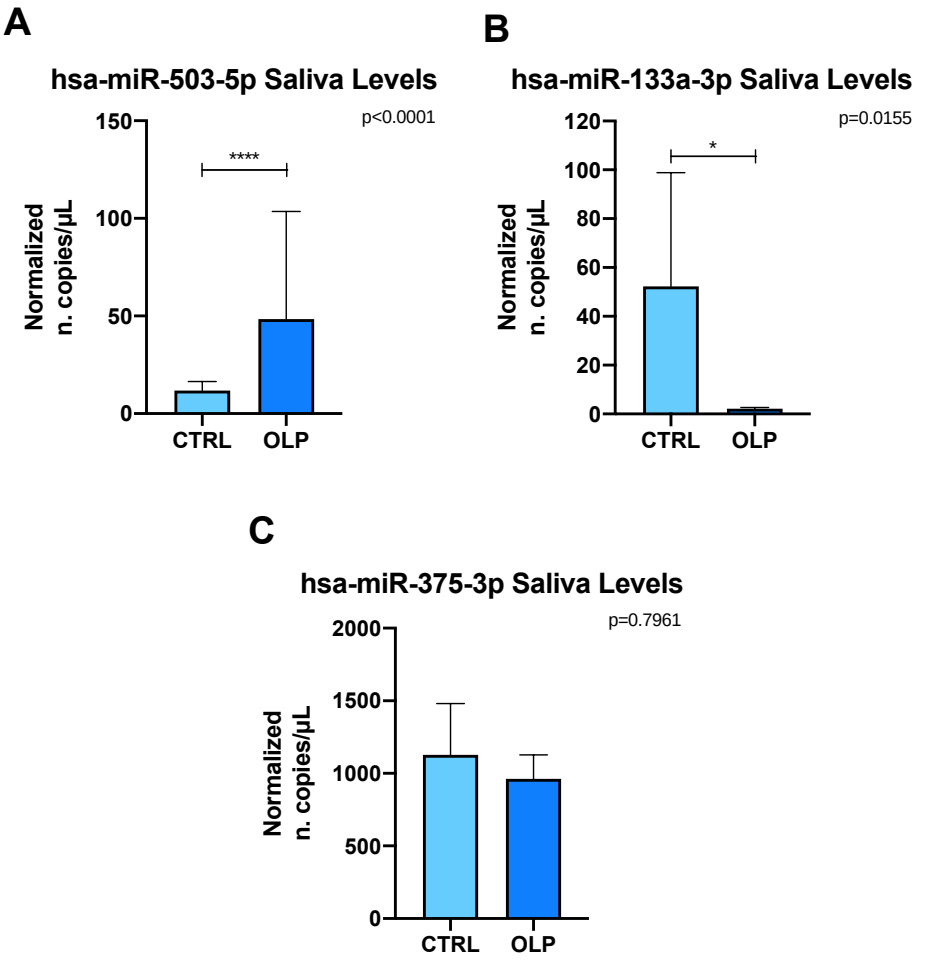

Figure 6. Expression levels of the three selected miRNAs, hsa-miR-503-5p **(A)**, hsa-miR-133a-3p **(B)** and hsa-miR-375-3p **(C)**, in oral lichen planus patients and normal controls. Student t-test: * $p<0.05$, **** $p<0.0001$.

These preliminary results obtained revealed the higher specificity of the ddPCR analyses performed on saliva samples compared to serum samples. Indeed, the expression levels of salivary miRNAs were more concordant with the results obtained in the previous computational analyses.

To further validate the involvement of the three selected miRNAs in the malignant transformation of OLP lesions, the expression levels of miRNA were analyzed also in serum and saliva samples obtained also from oral cancer patients. These latter ddPCR analyses performed on serum samples demonstrated heterogeneous results as the only significant data were obtained for the predicted up-regulated hsa-miR-503-5p, however, the levels of this miRNA were increased in OLP patients and then again decreased in oral cancer patients ($p=0.0218$; Figure 7A). As regards the two predicted down-regulated

hsa-miR-133a-3p and hsa-miR-375-3p no significant results were obtained, however a trend decrement and increment was observed for hsa-miR-133a-3p and hsa-miR-375-3p, respectively (Figure 7B and Figure 7C).

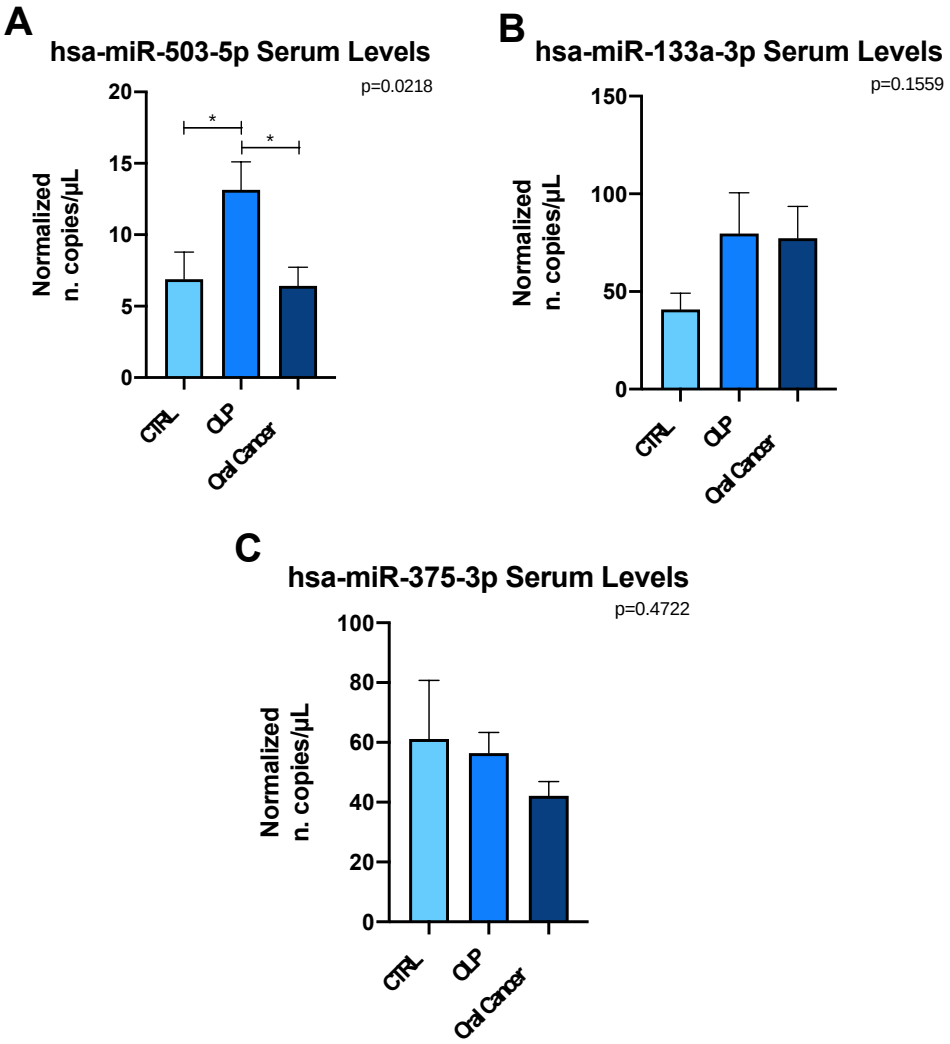

Figure 7. Expression levels of the three selected miRNAs, hsa-miR-503-5p **(A)**, hsa-miR-133a-3p **(B)** and hsa-miR-375-3p **(C)**, in serum samples of normal controls, oral lichen planus patients and oral cancer patients. Kruskal-Wallis test (plus Dunn's post-hoc multiple comparison test): * $p<0.05$.

Again, the results obtained by analyzing the miRNA expression levels in saliva samples of oral cancer, OLP and normal individuals revealed more reliable results (Figure 8). In this case the statistical analyses demonstrated a strong increment of hsa-miR-503-5p in both OLP and oral cancer compared to normal controls ($p=0.0009$; Figure 8A) as well as a strong statistical decrement of hsa-miR-133a-3p saliva levels in pre-neoplastic and neoplastic patients compared to

normal individuals (p=0.0023; Figure 8B). No significant variation of hsa-miR-375-3p expression levels was observed (Figure 8C).

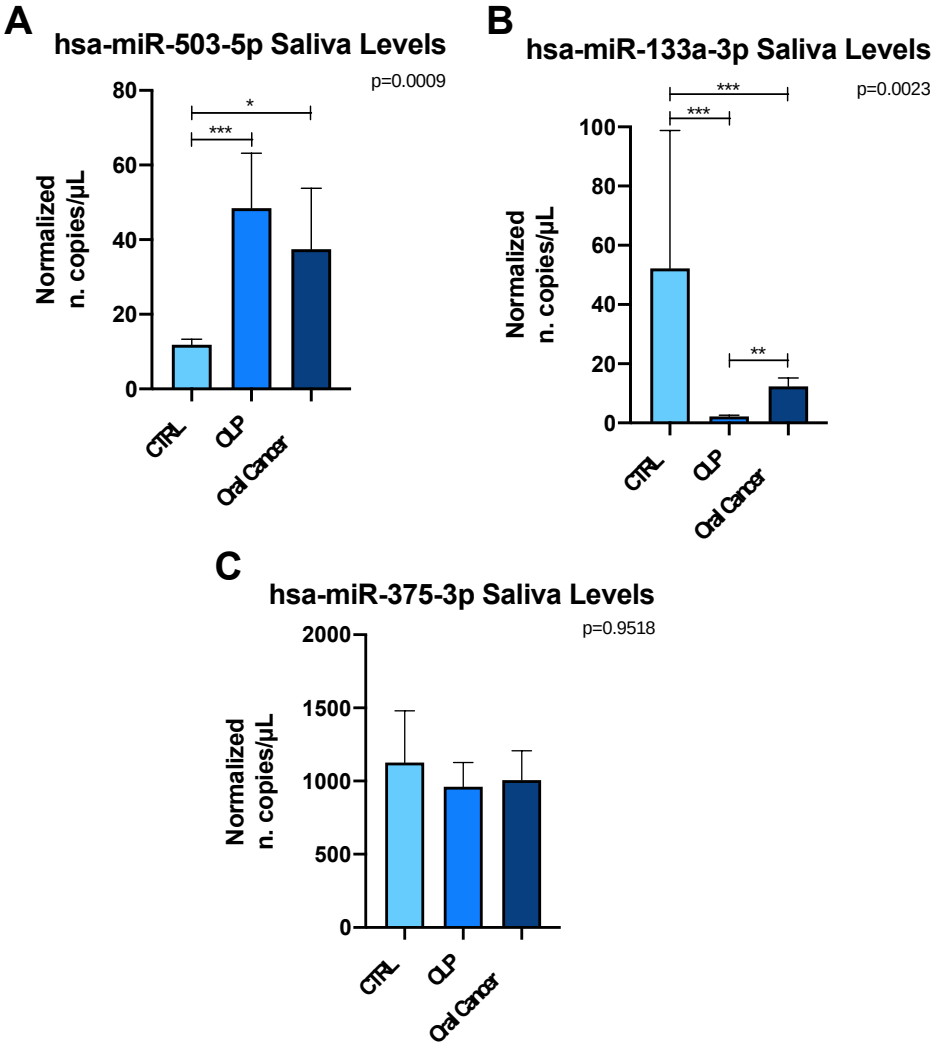

Figure 8. Expression levels of the three miRNAs, hsa-miR-503-5p **(A)**, hsa-miR-133a-3p **(B)** and hsa-miR-375-3p **(C)**, in saliva samples of normal controls, oral lichen planus patients and oral cancer patients. Kruskal-Wallis test (plus Dunn's post-hoc multiple comparison test): * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Overall, the ddPCR results here obtained demonstrated that the evaluation of the selected miRNAs in saliva samples of individuals at risk for oral cancer may predict the risk of development of this tumor. In addition, comparing the data obtained from both serum and saliva samples it was demonstrated the higher potential of saliva as a non-invasive sample useful to improve the diagnostic strategies available for oral cancer.

4.3 Diagnostic Potential of the Three Validated microRNAs

To better evaluate the diagnostic potential of the three selected miRNAs, Receiver Operating Characteristic curve (ROC) analyses were performed.

ROC analyses performed for the serum and saliva miRNA expression levels observed in normal individuals and OLP patients showed that the evaluation of hsa-miR-503-5p is able to discriminate between normal samples and OLP with good sensitivity and specificity rates. In particular, statistically significant results were obtained for hsa-miR-503-5p in both serum (p=0.0192) and saliva samples (p<0.0001) (AUC=0.7857, IC 95% from 0.5792 to 0.9922 and AUC=0.9786, IC 95% from 0.9329 to 1.000, respectively) with a sensitivity rate of 92.86% and a specificity rate ranging from 70.00% to 91.11%, respectively for serum and saliva samples (Figure 9). As previously mentioned, the detection of hsa-miR-503-5p in saliva samples has a higher diagnostic value compared that performed in serum samples. As regards, hsa-miR-133a-3p in saliva samples, a statistically significant ROC curve was obtained (p=0.0164; AUC=0.7929, IC 95% from 0.5976 to 0.9881) demonstrating a sensitivity rate of 92.86% and a specificity rate of 60.00% (Figure 9).

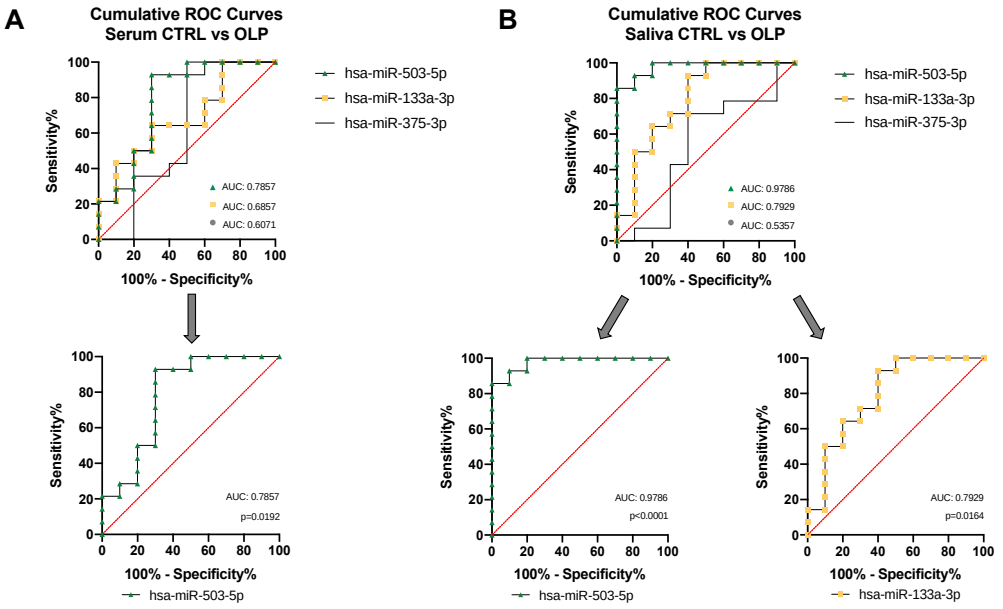

Figure 9. ROC analyses performed for the serum **(A)** and saliva **(B)** miRNA expression levels observed for normal individuals and oral lichen planus patients.

Considering only OLP and oral cancer patients, ROC analyses revealed that hsa-miR-503-5p expression levels in serum samples have a good prognostic value in

predicting the malignant transformation of OLP towards oral cancer (p=0.0224) (AUC=0.7857, IC 95% from 0.5861 to 0.9710) with a good sensitivity rate of 90.00% but a low specificity rate of 50%, (Figure 10). As regards saliva expression levels, ROC analyses revealed that hsa-miR-133a-3p expression levels have an excellent diagnostic value for the malignant transformation of OLP (p=0.0016) (AUC=0.8857, IC 95% from 0.7102 to 1.000) with an excellent sensitivity rate of 90.00% and an excellent specificity rate of 85.71%, (Figure 10).

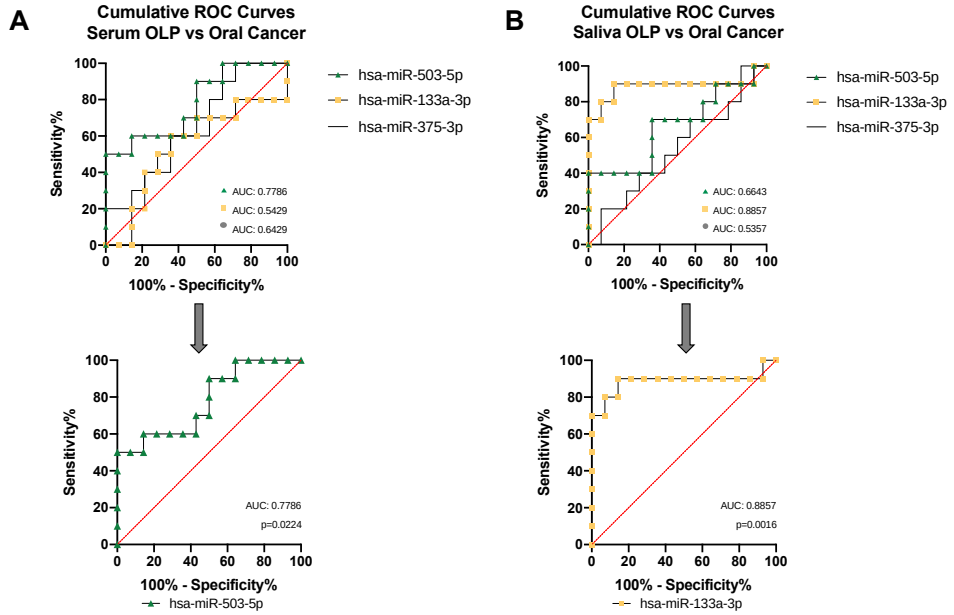

Figure 10. ROC analyses performed for the serum **(A)** and saliva **(B)** miRNA expression levels observed for oral lichen planus patients and oral cancer patients.

4.4 Functional and Prognostic Roles of the Three miRNAs Validated

In order to establish the functional roles of the three miRNAs here validated, COSMIC and mirDIP analyses were performed. Through COSMIC the ten most altered genes in oral cancer were identified. Subsequently, mirDIP analysis revealed the interaction levels of hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p with the ten altered genes (Figure 11).

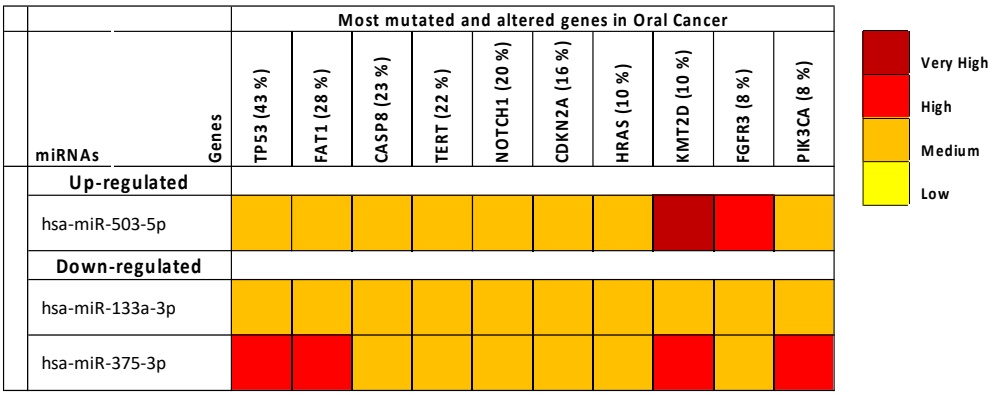

Figure 11. Interaction levels between the three analyzed miRNAs and the most altered genes in oral cancer.

In particular, COSMIC analyses highlighted that the ten most altered genes in oral cancer are *TP53* (43%), *FAT1* (28%), *CASP8* (23%), *TERT* (22%), *NOTCH1* (20%), *CDKN2A* (16%), *HRAS* (10%), *KMT2D* (10%), *FGFR3* (8%) and *PIK3CA* (8%). Of these genes, the majority showed moderate/high interaction levels with all the three selected miRNAs. In particular, hsa-miR-375-3p showed the highest interaction levels with the selected genes and in particular with *TP53*, *FAT1*, *KMT2D* and *PIK3CA*. Similarly, hsa-miR-503-5p showed very high and high interaction levels with *KMT2D* (Figure 11). Of note, KMT2D is an oncosuppressor gene whose inhibition mediated the over-expressed miRNA can reduce the protective action of this gene in oral cancer patients.

To better clarify the molecular pathways altered by the three miRNAs, the DIANA-mirPath analysis identified the most altered genes and pathways.

In particular, the selected miRNAs were able to alter 23 different pathways and a total of 201 genes. Several of these pathways are strongly altered in different tumors (Table 5).

Table 5. Genes and pathways modulated by the three selected miRNAs

N.	KEGG pathway	p-value	N. genes	N. miRNAs
1	Glycosaminoglycan biosynthesis - chondroitin sulfate (hsa00532)	4.77E-23	2	1
2	Viral carcinogenesis (hsa05203)	1.03E-06	17	3
3	Prostate cancer (hsa05215)	1.03E-06	11	3
4	p53 signaling pathway (hsa04115)	1.47E-06	9	3
5	Pathways in cancer (hsa05200)	1.52E-05	19	3
6	Colorectal cancer (hsa05210)	2.33E-05	7	3
7	Protein processing in endoplasmic reticulum (hsa04141)	1.91E-04	14	3
8	Small cell lung cancer (hsa05222)	1.91E-04	8	3
9	Apoptosis (hsa04210)	3.46E-04	7	2
10	mTOR signaling pathway (hsa04150)	4.08E-04	7	2
11	Hepatitis B (hsa05161)	4.53E-04	12	3
12	Non-small cell lung cancer (hsa05223)	4.85E-04	5	3
13	Pathogenic Escherichia coli infection (hsa05130)	8.92E-04	6	2
14	Cysteine and methionine metabolism (hsa00270)	1.54E-03	5	1
15	Cell cycle (hsa04110)	3.40E-03	11	2
16	PI3K-Akt signaling pathway (hsa04151)	4.63E-03	17	3
17	Focal adhesion (hsa04510)	9.51E-03	12	3
18	Glycosaminoglycan biosynthesis - heparan sulfate / heparin (hsa00534)	1.38E-02	2	1
19	Pancreatic cancer (hsa05212)	1.51E-02	6	3
20	Ether lipid metabolism (hsa00565)	4.40E-02	4	1
21	VEGF signaling pathway (hsa04370)	4.40E-02	5	2
22	Cholinergic synapse (hsa04725)	4.40E-02	8	2
23	Toxoplasmosis (hsa05145)	4.40E-02	7	2

Of the cancer-related pathways, the most altered were *Pathways in cancer* (hsa05200) (19 modulated genes), *PI3K-Akt signaling pathway* (hsa00565) (17 modulated genes) and *Viral carcinogenesis* (hsa05203) (17 modulated genes) all molecular pathways involved in the development of tumors when altered. Indeed, among the most altered genes there were *MAPK1* (#10), *CCND1* (#10), *AKT3* and *PIK3CA* (#9), *PIK3CB* (9), *NRAS* (#9), *BRAF* (#7), etc., all genes widely reported in the literature to be involved in the development of pre-cancerous lesions and in the neoplastic transformation of cells.

The DIANA-miRPath results have been obtained taking the data from Tarbase repository, therefore, to better identify the genes targeted by the three miRNAs hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p further prediction tools were used. In this context, the results obtained with the miRTargetLink Human tool showed a network of 21 genes targeted at least by two of the three selected miRNAs (Figure 12).

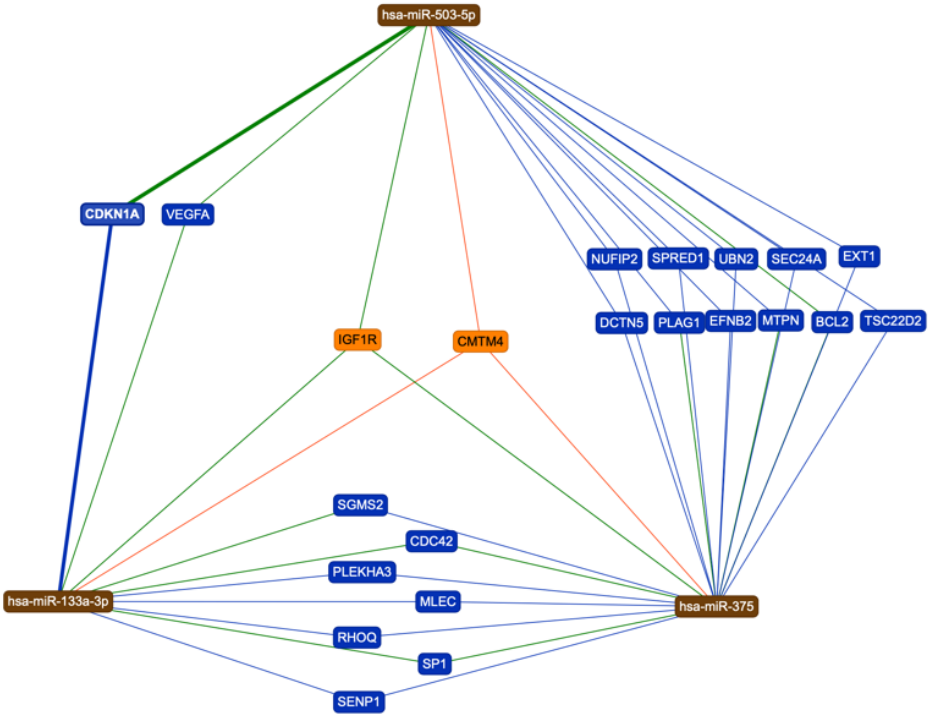

Figure 12. miRTargetLink Human analysis performed to identify the main genes targeted by hsa-miR-133a-3p and hsa-miR-375-3p.

Of the miRNA-targeted genes identified through miRTargetLink Human, *IGF1R* and *CMTM4* were concomitantly targeted by all the three selected miRNAs, suggesting that these genes are strongly subjected to epigenetic modulation mediated by miRNAs.

Noteworthy, the two down-regulated miRNAs hsa-miR-133a-3p and hsa-miR-375-3p are able to target seven genes, i.e. *SGMS2*, *PLEKHA3*, *RHOQ*, *CDC42*, *SP1* and *SENP1*, suggesting that these genes could be over-expressed in oral cancer as a consequence of miRNA dysregulation. Other results are related to the two genes targeted by hsa-miR-133a-3p and hsa-miR-503-5p (*CDKN1A* and *VEGFA*), and the 11 genes targeted by hsa-miR-375-3p and hsa-miR-503-5p (*NUFIP2*, *SPRED1*, *UBN2*, *SEC24A*, *EXT1*, *DCTN5*, *PLAG1*, *EFNB2*, *MTPN*, *BCL2* and *TSC22D2*).

To establish the interaction network existing among the miRNA-targeted genes identified through miRTargetLink Human STRING was used. The protein-protein interactions (PPI) network obtained by STRING revealed that all the 21 targeted genes were strongly interconnected. Among these, main nodes were

represented by *IGFR1*, *CDC42*, *VEGFA* and *CDKN1A* which showed the highest number of interconnections (Figure 13).

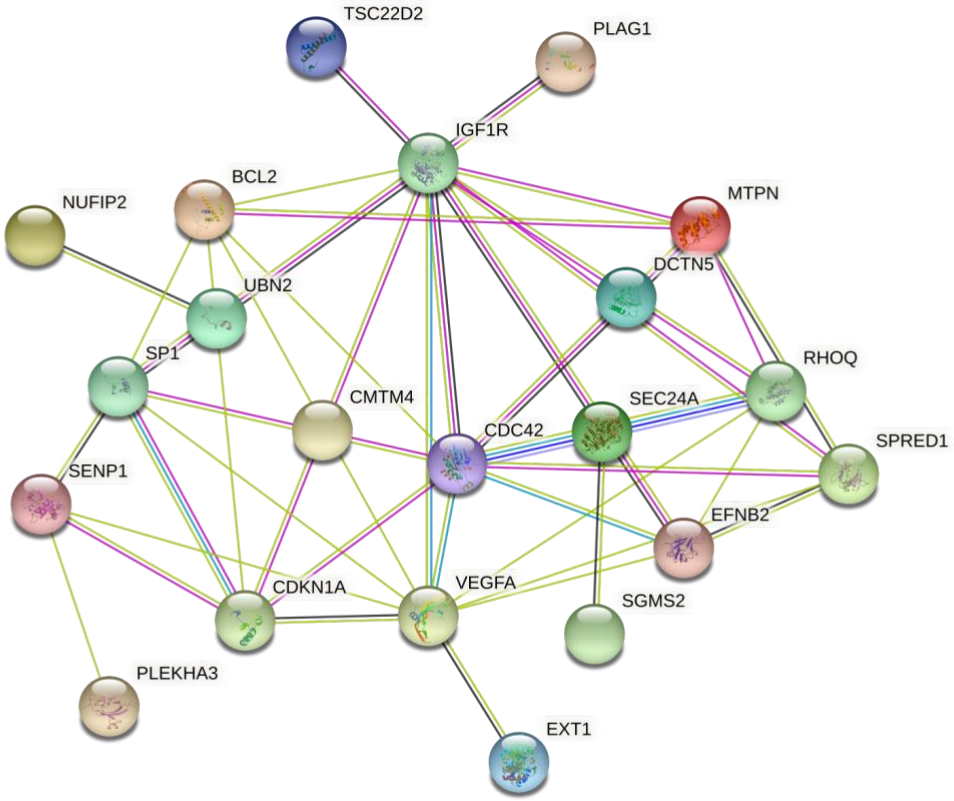

Figure 13. STRING protein-protein interaction network of 75 genes targeted by both hsa-miR-133a-3p and hsa-miR-375-3p.

These data further confirm the notion that the miRNAs identified may synergistically act mediating the dysregulation of their targeted genes in turn responsible for oral cancer development.

Besides these analyses, to clarify the functional roles of these 21 miRNA-targeted genes, GO PANTHER enrichment analysis was performed.

As regards the molecular functions where the 21 miRNA-targeted genes are involved, the GO PANTHER analysis revealed how these are mainly involved in binding activities (13 genes) and catalytic activities (6 genes) (Figure 14).

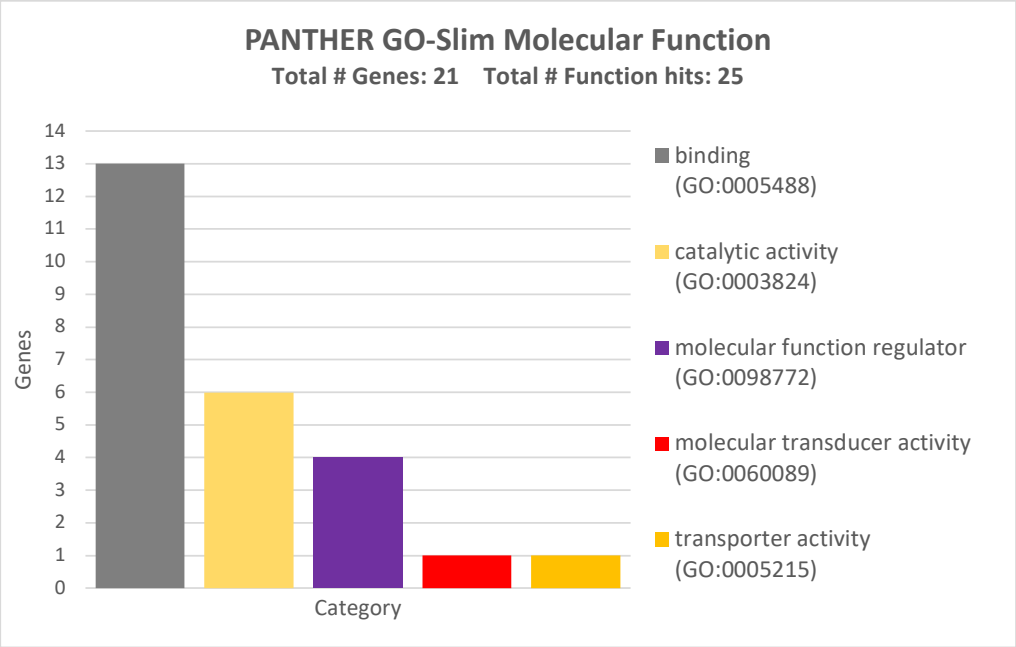

Figure 14. The GO PANTHER analysis revealed the "Molecular Functions" of the 21 genes targeted by hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p.

Regarding the "Biological Process" category, the genes targeted by the three miRNAs are mainly involved in the regulation of cellular processes (15 genes), in biological regulation (11 genes) and metabolic, signaling and response to stimuli processes (8 genes) (Figure 15).

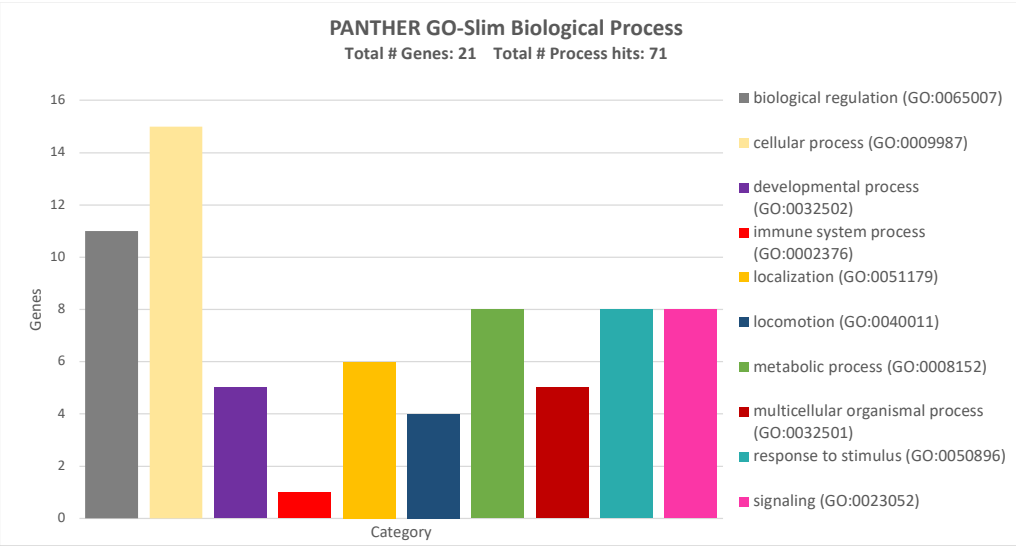

Figure 15. The GO PANTHER analysis revealed the "Biological Processes" of the 21 genes targeted by hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p.

Finally, the analysis of the protein classes of the 21 genes demonstrated that most genes encode for modulator of protein-binding activity (3 genes) and for gene-

specific transcriptional regulators, intracellular signal molecules, membrane traffic proteins and scaffold/adaptor protein (2 genes each) (Figure 16).

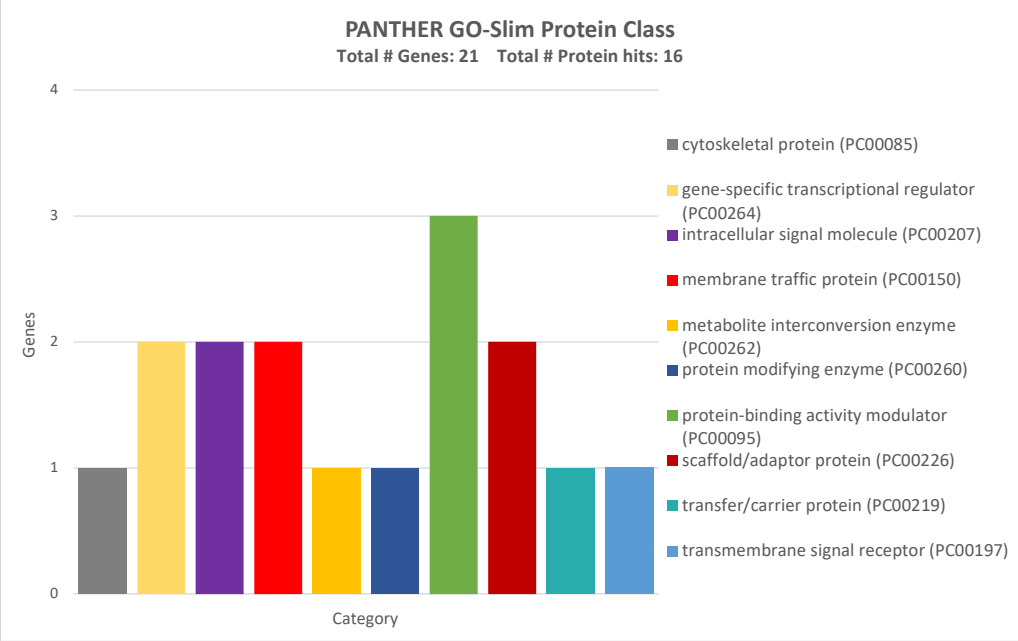

Figure 16. The GO PANTHER analysis revealed the "Protein Class" of the 21 genes targeted by hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p.

To further strengthen the experimental and computational results obtained, the dysregulation of the expression levels of the 21 miRNA-targeted genes in oral cancer was evaluated through GEPIA. As shown in Figure 17, only the genes *IGF1R*, *VEGFA*, *EXT1*, *TSC22D2* and *SENP1* were significantly altered in TCGA oral cancer samples compared to TCGA and GTEx normal controls (Figure 17).

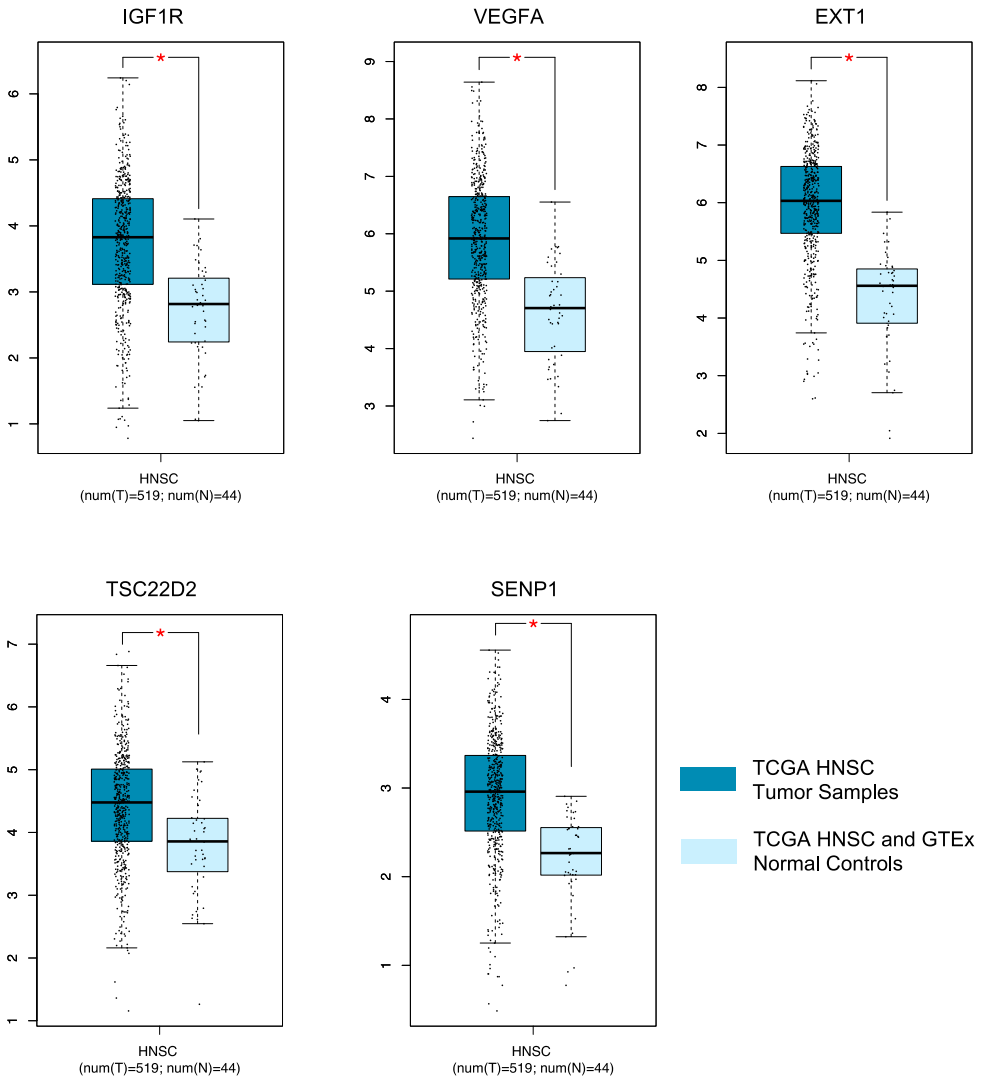

Figure 17. GEPIA analysis of the miRNA-targeted gene dysregulation in TCGA HNSC oral cancer samples compared to TCGA HNSC and GTEx normal controls. The p-value threshold was set at 0.01 (*=p<0.01). The relative expression levels were first log2(TPM+1) transformed and the log2FC was defined as median (Tumor)—Median (Normal), where TPM is the transcript count per million.

Finally, the analysis of the TCGA HNSC miRNA expression levels and clinical characteristics data allowed us to establish the prognostic potential of the evaluation of hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p. The analysis of the expression levels of these three miRNAs according to the patients' stage demonstrated that all the three miRNAs were strongly dysregulated (Figure 18). In particular, the expression levels of the two predicted and validated down-regulated miRNAs hsa-miR-133a-3p and hsa-miR-375-3p

decrease significantly in the patients with more advanced tumors (p=0.0178 and p<0.0001, respectively) (Figure 18A and 18B) while the expression levels of hsa-miR-503-5p increased significantly in the most advanced tumors (Figure 18C).

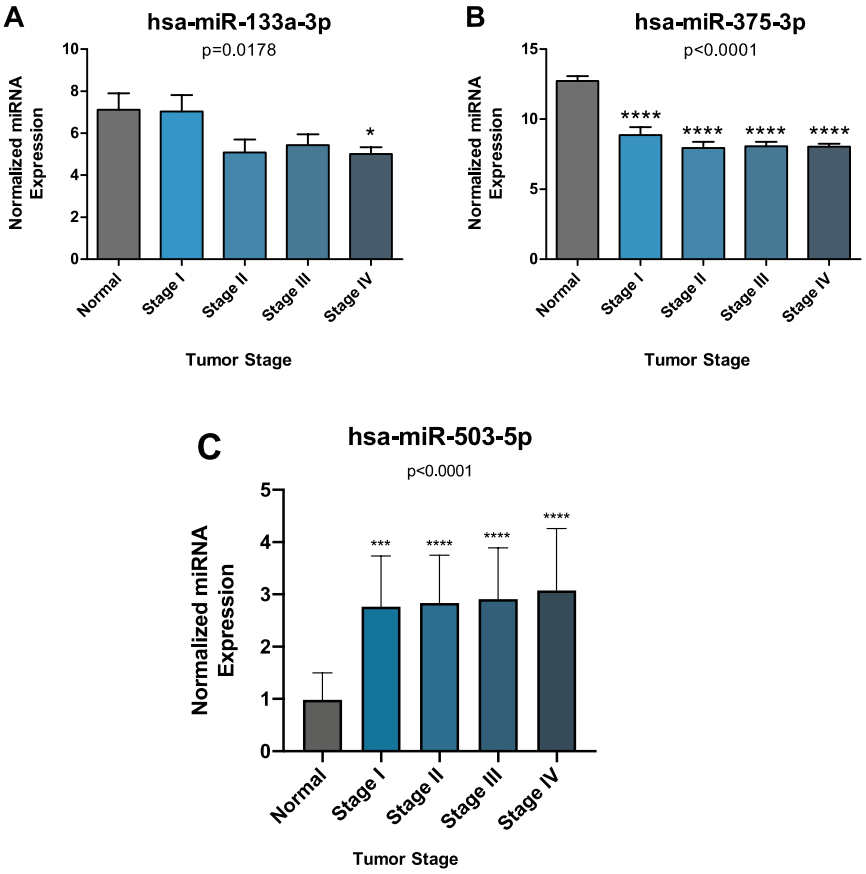

Figure 18. Expression levels of hsa-miR-133a-3p **(A)** and hsa-miR-375-3p **(B)** and hsa-miR-503-5p **(C)** in oral cancer patients at different stages according to HNSC TCGA data. *: p <0.05; ***: p<0.001; ****: p <0.0001.

Taking into account the presence of lymph node metastases, the expression levels of hsa-miR-503, hsa-miR-133a-3p and hsa-miR-375-3p do not vary significantly (Figure 19).

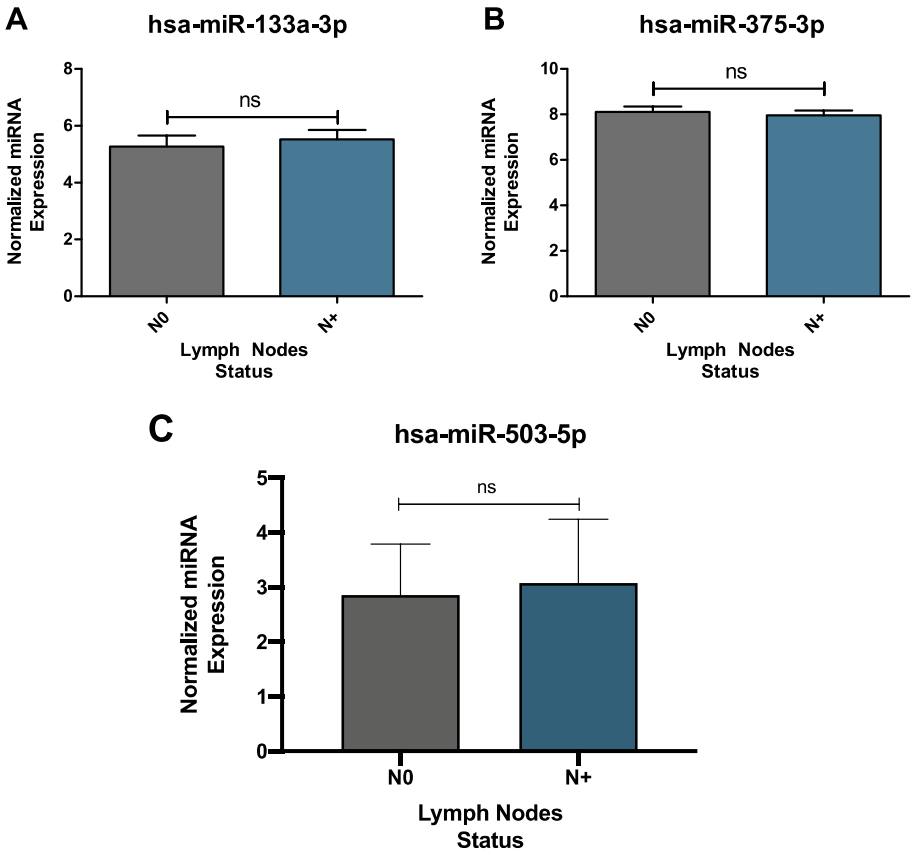

Figure 19. Expression levels of hsa-miR-133a-3p **(A)** and hsa-miR-375-3p **(B)** and hsa-miR-503-5p **(C)** in oral cancer patients according to TCGA HNSC lymph node status.

Overall, these data suggest that the concomitant evaluation of the expression levels of these three miRNAs may be useful for the early diagnosis of OLP lesions and for the prediction of oral cancer development.

5. DISCUSSION

Despite the advancement in the diagnosis and treatment of tumors, the prognosis of patients affected by oral cancer is still negative due to the lack of effective diagnostic and prognostic biomarkers and consequently the delay in the diagnosis or the misdiagnosis of pre-cancerous oral lesions. Of note, the current strategies adopted for the diagnosis of tumors are based on the detection of cancer biomarkers like EAA, CA19-9, CA125 or serum and salivary pro-inflammatory cytokines including IL-6 and IL-8. However, the sensitivity and specificity rates of these biomarkers are very low and lead to a high rate of false-positive and false-negative results (123-125). Therefore, it is evident how the misdiagnosis of oral lesions is responsible for the diagnosis of tumors in an advanced stage with a consequent high probability of death.

On these bases, there is an urgent need to discover novel promising biomarkers for the early diagnosis of oral cancer and for the prognosis of patients to better manage this disease. In this context, several studies have proposed the analysis of the expression levels of miRNAs as a novel promising diagnostic strategy for the early diagnosis of tumors (126,127). In particular, the discovery of miRNAs easily detectable in the bloodstream and other biological fluids, has prompted the scientific community to question if the evaluation of these biomarkers in serum, saliva, urine, etc., could be used for the early diagnosis of tumors and to establish the prognosis of oral cancer patients.

Starting from this research question, the group of Maxillo-facial surgery together with the Experimental Oncology Laboratory of the University of Catania was the first to fully evaluate all the bioinformatics data available for oral cancer related to miRNA expression levels in tumor and normal samples. In particular, the bioinformatic data contained in GEO DataSets and TCGA databases were analyzed (128).

Through these computational analyses, a preliminary panel of miRNAs significantly dysregulated in oral cancer was identified. By comparing the lists of dysregulated miRNAs obtained from the analysis of miRNA expression data contained in the GEO DataSets and TCGA HNSC databases, it was possible to computationally identify 11 dysregulated miRNAs in oral cancer, of which four

were up-regulated and seven down-regulated. For all these 11 computationally selected miRNAs, further bioinformatics analyses were performed in order to establish the real involvement in cancer development and the functional roles of these miRNAs in oral cancer. These further analyses allowed us to select two up-regulated miRNAs, hsa-miR-503-5p and hsa-miR-196a-5p, and two down-regulated miRNAs, hsa-miR-133a-3p and hsa-miR-375-3p, for the validation analyses performed on human samples.

In particular, the expression levels of these four selected miRNAs were validated in a pilot cohort of ten oral cancer patients, ten healthy controls and 14 OLP patients enrolled at the Maxillofacial Unit of the San Marco Hospital of Catania and available in the biobank of the Experimental Oncology Laboratory of the University of Catania.

Noteworthy, the validation analyses were performed by using the high-sensitive ddPCR amplification system which ensures the effective detection of low expressed targets as circulating miRNAs thus representing the best method for the detection of miRNAs in liquid biopsy samples. Therefore, the expression levels of miRNAs were evaluated in both serum and saliva samples obtained from each individual enrolled in the study.

According to the computational prediction analyses, ddPCR results showed hsa-miR-503-5p and hsa-miR-133a-3p have a good diagnostic value when evaluated in both serum and saliva samples. Specifically, the miRNA expression data obtained in saliva samples were more consistent and robust compared to those observed in serum samples as high significant difference in the expression levels of both hsa-miR-503-5p and hsa-miR-133a-3p between OLP patients and controls was obtained ($p<0.0001$ and $p=0.0155$, respectively). Further ddPCR analyses evaluating the expression levels of the three selected miRNAs among OLP and oral cancer patients as well as normal controls revealed that both serum and saliva hsa-miR-503-5p expression levels may be considered as good biomarkers for the diagnosis of OLP and oral cancer. Again, these results demonstrated the superiority of saliva samples for which the data showed a higher statistical significance.

These results were further corroborated by the results obtained with ROC analysis which confirmed that the analysis of the expression levels of hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p, alone or in combination, can discriminate between normal and tumor samples with sensitivity and specificity rates ranging from 92-90% and 91-50%, respectively, suggesting how the evaluation of these miRNAs in serum and saliva samples of patients with suspicious lesions may be helpful for the early identification of malignant oral lesions.

The results here obtained are in line with other scientific findings demonstrating the involvement and diagnostic potential of both hsa-miR-133a-3p and hsa-miR-375-3p in different tumors, including colorectal cancer, gastric cancer, hepatocellular carcinoma, prostate cancer, etc. (129-133).

After the ddPCR results, further bioinformatics analyses were performed to establish the prognostic role of the miRNAs here identified as well as their functional roles in oral cancer development and progression. For this purpose, miRNA-gene interaction analyses were performed using miRTargetLink Human and the TCGA HNSC database. These further analyses revealed that the three validated miRNAs are able to concomitantly target a total of 21 genes (which shared two or three miRNAs). Of these genes, some are known to be involved in the development and progression of different tumors through the alteration of cell proliferation and apoptosis mechanisms, like IGF1R, VEGFA, BLC2, also involved in the development of oral cancer (134-136).

In addition, all the 21 miRNA-targeted genes were involved in a complex protein-protein interaction network as demonstrated by the STRING analysis further suggesting how these three miRNAs can mediate the modulation of these genes involved in oral cancer development. This notion was further corroborated by the GO PANTHER results highlighting the involvement of these 21 genes in molecular functions and biological processes underlying the development of tumors.

Finally, the GEPIA analysis and the evaluation of the expression levels of miRNAs according to the clinical-pathological features of oral cancer patients contained in the TCGA HNSC database demonstrated that five out of the 21

miRNA-targeted genes were significantly dysregulated in oral cancer, IGF1R, VEGFA, EXT1, TSC22D2 and SENP1, suggesting how the epigenetic modulation of these genes mediated by the three selected miRNAs may be responsible for the development of tumor-initiating processes. In addition, the evaluation of the expression levels of these three miRNAs in patients with a confirmed diagnosis of oral cancer can be predictive for the evolution of the disease as the expression levels of hsa-miR-503-5p increased significantly according to tumor stages while the expression levels of hsa-miR-133a and hsa-miR-375-3p decreased significantly according to the same patients' characteristics.

Therefore, the evaluation of these three miRNAs could be useful for both diagnostic and prognostic purposes in the management of oral cancer.

6. CONCLUSIONS

The results of the present study represent the first example of computational and experimental approaches aimed at assessing the diagnostic and prognostic potential of circulating miRNAs detected in both serum and saliva samples for the management of oral cancer.

To the best of our knowledge, this is the first study combining bioinformatics skills and advanced molecular approaches for the identification and validation of miRNAs as biomarkers for both oral lichen planus and oral cancer lesions. Indeed, through the use of the high-sensitive ddPCR amplification system the miRNAs computationally identified were validated on liquid biopsy samples obtained from healthy controls, OLP patients and oral cancer patients. In particular, ddPCR analyses revealed that the evaluation of miRNAs in both serum and saliva samples could be useful to early detect oral malignant transformation highlighting how the saliva is a better sample compared to serum. Therefore, these results strongly encourage the adoption of liquid biopsy and ddPCR for the non-invasive and effective diagnosis of oral cancer in case of suspicious lesions or in individuals at risk for this tumor. Overall, through the methodologies here adopted, a panel of three miRNAs, hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p, was validated for both diagnostic and prognostic purposes.

Although validated in a preliminary case series, this study represents the starting point for the development of novel diagnostic strategies based on the use of ddPCR for the analysis of liquid biopsy samples and the detection of miRNAs, with diagnostic and prognostic potential for the management of oral lesions. However, further experimental and functional studies on a large number of samples are needed to assess the expression levels of these miRNAs and to better validate their predictive role as biomarkers for oral cancer.

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