UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN SCIENZE DELLA VITA E BIOTECNOLOGIE

XXXIIICICLO

"Analysis of LncRNAs expression profile in oral squamous cell carcinoma"

"Analisi del profilo di espressione di LncRNA nel carcinoma a cellule squamose del cavo orale"

Docente guida: Prof. Lucia Tettamanti

Tesi di dottorato di:

Fabio Croveri

Matr. 705364

Dip. Biotecnologie e Scienze della Vita -Università degli Studi dell'Insubria

Anno accademico 2019-2020

1. INTRODUCTION

The role of the Dentist and, in particular, the role of the Oral Medicine specialist as a front-line sentinel in the interception of oral cancer has been consolidated since long time. However, over the last decade there have not been any recent developed diagnostic strategies that can facilitate neither a more precise general framework nor a definition of the degree of local and systemic involvement, as well as the type of behavior.

Head and neck cancer (HNC), is the sixth most common type of cancer in the world and accounts for approximately 550,000 cases per year [1]. HNC encompasses a wide range of malignancies arising from the head and neck region, including the oral district, the oropharynx, and larynx. More than 90% of HNCs are squamous cell carcinomas (SCCs), which frequently develop from the mucous surfaces of the mouth [2].

Oral squamous cell carcinoma (OSCC), characterized by differentiation and a tendency to metastatic lymph node spread [3], is the sixth most common cancer

worldwide with over 200,000 new diagnoses every year globally.

From a topographical point of view, it can be divided into three main sub-sites: buccal mucosal SCC (BMSCC), tongue SCC (TSCC) and labial SCC (LSCC) [4], [5]. The morbidity and mortality rates in males are 6.6/100,000 and 3.1/100,000respectively, while in females the same percentages are 2.9/100,000 and 1.4/100,000 [6]. Furthermore, the incidence of OSCC is increasing among young white individuals aged 18 to 44 years, particularly among white women [7]. Due to the frequency of exposure to risk factors in the population, the low cure rate and high mortality, OSCC represents a global public health problem with a large individual and socioeconomic burden.

This type of cancer is very common in South-Central Asia, being the most common cancer in men in high-risk countries [8]. It is reported in the literature that in South Asian countries such as India, Sri Lanka, Pakistan and Bangladesh a quarter of new cancer cases are diagnosed as OSCC, being the third most common type of cancer [9]. Many risk factors have been linked to the

development of OSCC, most notably Rodriguez et al. showed in their study that, in terms of risk attributable to the population, tobacco smoking accounted for 77% of all oral and pharyngeal cancers, alcohol consumption for 52% and the combination of the two for 83%. As for the diet, the risks attributable to the population estimated were 52% for the low vegetable content, 12% for the low fruit content, 26% for the low β-carotene intake. The combination of tobacco, alcohol and low vegetable consumption accounted for 85% of all cases in this population [10].

When diagnosed at an advanced stage, treatment of OSCC involves particularly invasive procedures since, in most cases, the disease is fatal due to the high rate of early metastasis to regional cervical lymph nodes; treating these patients is a challenge for clinicians [11]. Early-stage OSCC, however, has a favorable prognosis and requires less aggressive treatment [12]. The 5-year overall survival rate for OSCC is less than 50% and has improved only modestly over the past 2 decades despite the dramatic improvement in treatment modalities [13].

Although tumor-node-metastasis (TNM) based on neoplastic staging is routinely used to predict tumor behavior and, consequently, to inform about the choice of treatment strategies of OSCC, patients with the same TNM stages may have non-overlapping clinical evolutions and a significantly different survival time [14]. These inconsistencies require the development of new and more in-depth prognostic techniques and tools that can be used in the management of OSCC.

For these reasons, the search for biomarkers which could be used for diagnosis and prognosis represents a growing hope to provide doctors with new tools for the treatment of the disease.

The term Biomarker refers to an indicator, which can be measured, and which can be linked to a specific condition. Several definitions have been proposed for biomarkers in the literature. The Biomarkers Definition Working Group (formed by the US National Institutes of Health (NIH) and the US Food and Drug Administration (FDA), academia and industry) defined the biomarker as "a feature that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic

processes or pharmacological responses to a therapeutic intervention”[15]. With the advent of the genomic era, in April 2008, the FDA published - in one of its documents - "Guidance for Industry" specific definition of a genomic biomarker as "a measurable characteristic of DNA and/or RNA that is an indicator of normal biological processes, pathogenic processes, and/or response to therapeutic or other interventions" [16]. More recently, Anderson and Kodukulahave provided some definitions of different types of biomarkers (e.g., surrogate, clinical, diagnostic, prognostic, predictive, pharmacodynamic, efficacy, and toxicity/safety endpoints in their review of role of biomarkers in pharmacology and drug discovery) [17]. All these definitions specify the requirements that a biomarker must possess, the different existingtypes, their potential role in the diagnosis and progression of the disease or in the control of the therapeutic response and their usefulness for the evaluation of new chemical entities as potential guiding therapies [18].

Recently, due to their relative stability and abundance, non-coding RNAs have emerged as useful biomarkers in

tumors, due to their diverse roles in many biological processes, from carcinogenesis, cell differentiation and metastasis. These could provide physicians with diverse information, from the biology of tumors to their correlation to clinical outcomes [19].

In this perspective, oral carcinogenesis is a multi-step process involving the development and progression of OSCC from normal mucosa or premalignant lesions [20]. This process has led not only to irreversible genetic changes in the DNA sequence, but also to epigenetic events orchestrated by key regulatory molecules [21], [22]. Recently, the role of non-coding RNAs, including long and microRNAs, has acquired considerable importance in the epigenetics of carcinogenesis treatment for this type of disease must challenge the significant discrepancies in therapeutic response in individuals. Thus, the discovery and validation of new biomarkers such as non-coding RNAs will contribute to more accurate treatment monitoring and the development of personalized therapies, both with a direct impact on patients' quality of life [23].

1.1 Squamous cell carcinoma of the oral cavity (OSCC) causative factors

More than 90% of malignant neoplasms of the upper aerodigestive tract are represented by squamous cell carcinomas (SCC) with common causative factors [24]. The keratinocyte of the oral mucosa represents the cellular structure from which this type of neoplasm originates. Moreover, a spontaneous DNA mutation in this cellular structure - often associated with the co-presence of physical, chemical or microbial mutagenic agents - represents the causal biological factor. Following these initial genomic alterations, oral keratinocytes can move towards a premalignant or potentially malignant profile and can associate with an altered cell proliferation, up to a condition of cellular autonomy and complete release from the cell cycle control systems, with subsequent invasion of frankly malignant cells through the epithelial basement membrane and their subsequent metastatic spread to the lymph node and/or systemic level.

The main agents associated with the genesis of an oral keratinocyte DNA mutation are represented by lifestyle related factors, although environmental and genetic predisposition aspects may represent additional risk factors related to the onset of the tumor [25].

As for the determining factors related to lifestyle, the role of tobacco use, and alcohol consumption certainly represents the most associatedelements with neoplastic onset, immediately followed by betel chewing and a diet associated with a high intake of fats and sugars. According to a study conducted by Petti, 25% of oral cancers are attributable to tobacco consumption (smoking and/or chewing), 7-19% to alcohol consumption, 10-15% to micronutrient deficiency in diets with reduced intake of fruit and vegetables, and more than 50% to the chewing of betel, even if referable only in geographic regions with a high prevalence of chewing [26].

With regard to environmental factors, viral infections, and in particular that of Human Papilloma Virus (HPV), find dissimilar results in investigations concerning its possible intervention in oral oncogenesis [27], while its

role in the genesis of oropharyngeal carcinomas HPV-related oncogenicity has been widely documented in the gynecological field where the virus is considered the main causative agent of cervical cancer [28]. The high-risk viral genotypes identified so far are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70 and the data relating to the scientific literature are constantly evolving [29]. After contagion, the life cycle of the virus is strictly dependent on the differentiation process of the keratinocyte, the target cell of the infection. It begins with the entry of the virus into the cells of the germinative layer of the epithelium, equipped with the specific α6 integrin receptor and marked proliferative activity, previously exposed to microabrasions of the epithelial lining. This aspect explains the particular susceptibility of some epithelial districts in which the basal and parabasal cells are constantly exposed to the environment (oropharyngeal mucosa at the tonsillar crypts, oropharynx).

Other types of infectious processes such as candidiasis and syphilis have been considered as possible authors in the onset of cancer and more recently in the

microbiological field the possible role of the oral bacterial flora has also been feared. Indeed, the discovery of a greater incidence of areas of dysplasia and esophageal cancer in people associated with poor oral hygiene and the fact that attention to dental health by the general population reduces the risk of oral cancer, has led to postulate a possible correlation between OSCC and oral microbial flora [30]. The presence of abundant accumulations of plaque above and below gingival and of periodontal disease conditions have in fact been correlated to the onset of pancreatic malignant neoplasms of the lung and even renal [31], hypothesizing a possible interaction with mutagen between the polymicrobial supragingival plaque and saliva [32].

Ultimately, various hereditary conditions such as xeroderma pigmentosum, Fanconi's anemia, dyskeratosis congenita and Li Fraumeni syndrome can play a favoring role in the development of OSCC [33].

In recent years, thanks also to the development of new genomic analysis and sequencing technologies, various genetic alterations characteristic of OSCC have been

attributed, mainly related to the overexpression of oncogenes and/or the silencing of tumor suppressor genes.

Using microarray-based gene expression analysis, Fialka F. et al noticed that 601 genes are differently regulated in cancerous tissue than adjacent contralateral mucosal controls. By comparing early-stage cancer samples with those from advanced disease, they measured the presence of 25 genes with important differences in terms of expression, in particular FMO2, CPA6, TNC, and SIAT1 genes were overexpressed in the early stages of the disease [34].

Chiang W.F. et al identified the mutation and overexpression of a gene - the epidermal growth factor receptor (EGFR) - in oral squamous cell carcinoma associated with the use of areca.

Significant increases in EGFR copy number were found in OSCC compared to adjacent oral mucosa, suggesting that genomic amplification could be a genetic basis for EGFR pathway activation in areca-associated OSCC [35].

According to the research conducted by Serefoglou Z. et al it has been evaluated that the presence of functional polymorphisms that influences the gene expression of the interleukins IL-4, -6, -8 and -10, as well as of the tumor necrosis factor-alpha (TNF -a), is strongly associated with an increased risk of OSCC [36].

It has also been assessed that the consumption of chewed tobacco induces TNF-a which, together with its receptors, is overexpressed in the OSCC. Single nucleotide polymorphisms (SNPs) in the TNF-a and TNF receptor genes can influence their expression and can be a determining factor for an increased susceptibility to OSCC related to smoked tobacco [37].

Coutinho-Camillo CM et al demonstrated, by analyzing by immunohistochemical investigations of 229 cases of OSCC, arranged in a tissue microarray, the possible involvement of proteins of the Bcl-2 family in the tumorigenesis of OSCC and suggest that the expression of molecules apoptosis could be used as a prognostic indicator for OSCC [38].

Recently, Chaudhary A. et al. have focused on matrix metalloproteinases (MMPs), which are enzymes that

degrade all components of the extracellular matrix, including collagen. They examined 362 patients with oral submucosal fibrosis (OSMF), head and neck injuries. They have concluded that the expression of the MMP-3 genotype associated with the 5A alleles may play an important role in the susceptibility of patients to develop OSMF, head and squamous cell carcinoma of the neck [39].

1.2 Epidemiology of oral squamous cell carcinoma (OSCC)

Squamous cell carcinoma (OSCC) is the most frequent malignant tumor of the oral cavity and oropharynx.
Epidemiological data indicate that, among all malignant tumors, it ranks sixth in the world average as per incidence [40].
In Italy, the average incidence is about 8 new cases per year for every 100,000 inhabitants among men and 3 new cases among women. The incidence rates are higher in the northern regions than in the central and southern ones.

In industrialized areas, in the Alpine valleys and especially in the north-east, oral cancer is widespread. The considerable variations in the regional incidence of oral cancer reflect differences related to local customs such as tobacco consumption and alcoholic beverages [41].

Oral cancer mainly affects subjects around the fifth-sixth decade of life, but in recent years the incidence in subjects under the age of 60 has drastically increased [42].

Prognosis in OSCC depends on tumor, treatment and patient factors. It is generally accepted that the prognosis is better in early cancers, especially in well-differentiated and non-metastatic ones: unfortunately, most OSCCs are diagnosed in an advanced stage of the disease.

The neoplasm spreads by forming predominantly lymphatic metastases in the lymph nodes of the neck. Metastasis can occur, albeit more rarely, even at a distance, especially in the lungs.

In recent decades, no improvement has been shown either in prognosis nor in therapy, so the differences in

the mortality rate between geographical areas and time periods could be attributed to variations in exposure to risk factors [43].

The average survival is 41% among men and 54% among women at 5-years survival rate [44].

Despite information and primary prevention programs throughout Europe, the analysis of mortality curves for oral and oropharyngeal cancer has shown stability in all countries for both men and women.

Although extremely expensive and characterized by a great expenditure of energy, the therapy is generally not very effective in the medium to long term given that 80% of stage III and IV patients die in the first 5 years; metastases recur in about 50% of patients.

Only if the cancer is diagnosed at an early stage, the 5-year survival rate reaches about 80-90% and the cost of therapy in human terms is lower.

For these reasons, early diagnosis plays a crucial role in improving the patient's prognosis.

1.3 Clinic and current therapies of squamous cell carcinoma

Squamous cell carcinoma is characterized by an early infiltrative tendency and a greater spread through the lymphatic route than the hematogenous one. Lymph node infiltration is influenced by the site of the neoplasm, its size, tumor histotype, histological differentiation, the anatomical structure of the affected organ and its lymphatic network, the relationships with nearby anatomical formations [45].

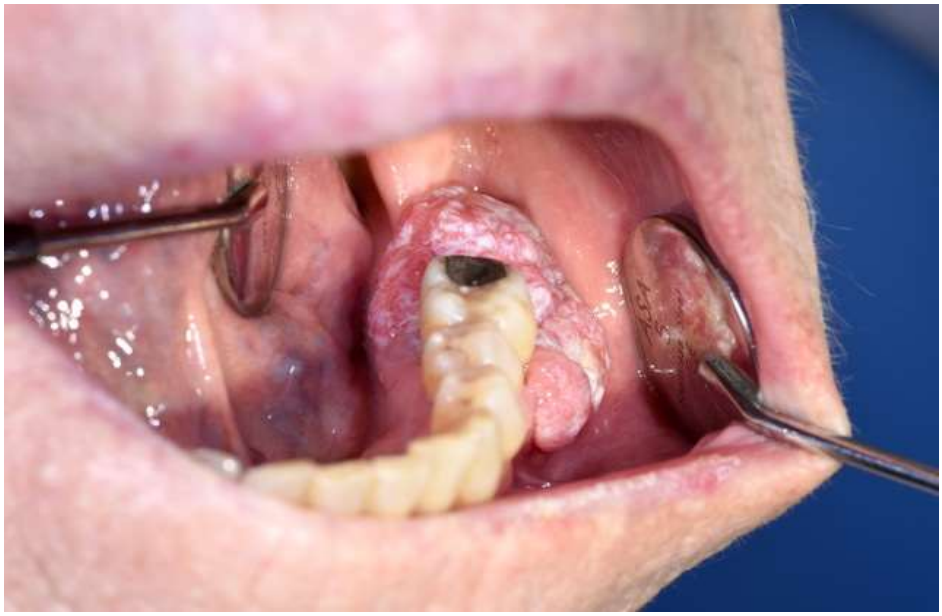


Fig.1. OSSC metastatic type

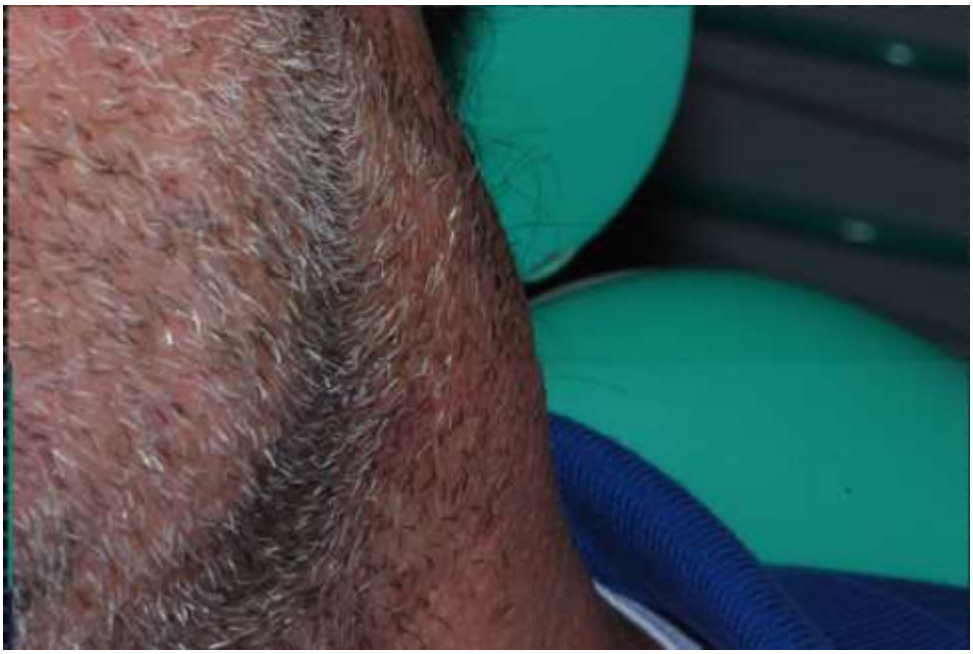


Fig.2. Lymph node infiltration

The presence of a solitary lump, associated with fixation in the periwound tissue, an indolent ulcer or a white or red lesion that has persisted for more than 3 weeks, the presence of an alveolus that does not evolve towards spontaneous healing or changes in sensitivity not referable to a specific cause can represent the initial manifestations and alarm bells connected to the onset of OSCC.

Visual inspection is the critical moment in the formulation of the diagnostic suspicion throughout inspection and palpation of the oral cavity and - possibly - fibroscopy of the VADS.

The clinical appearance of the carcinoma can be exophytic, endophytic (nodular or infiltrating), ulcerative with thickened edges or mixed exophytic-ulcerative [46].

Lymphatic diffusion is assessed through a palpatory clinical examination of the cervical-facial lymph node stations followed by imaging techniques (ultrasound, CT, MRI).

Clinical staging uses the TNM classification system of the International Union against Cancer (UICC) - tumor size (T), lymph node metastases (N) and distant metastases (M) which allows a correlation between neoplastic characteristics, the type of treatment and the survival rate [47].

However, the definitive diagnosis is histological: squamous cell carcinoma arises in the context of a dysplastic epithelium. It is characterized by nodules or cords of malignant epithelial cells which invade the spaces beyond the basement membrane [48].

Treatment of oral and oropharyngeal cancer uses the combination of demolition and reconstructive surgery, chemotherapy and radiotherapy.

Surgical therapy involves the resection of the primary tumor possibly associated with the removal of laterocervical lymphatic metastases and currently represents the most effective therapeutic response, especially if well-coordinated in the context of multimodal protocols.

Even though maxillofacial resective surgery represents an extremely invasive procedure due to the mutilation imposed on the patient by the surgical act, it still appears to be as the therapy of choice in the case of early injuries and in more advanced stages – both associated with or without lymph node removal [49].

Radiation therapy is often used as an adjuvant form of surgery, but in some inoperable cases it is considered the primary therapy. Fractionation is required to optimize the effect of the therapy on neoplastic tissue, given that ionizing radiation has a greater effectiveness of on the duplicating cells. Today it is suggested to administer a

dose of 2.0 Gy/fraction, for a total of 1000cGy/week, divided into one dose per day for five days.

The radio-treated patient in the cervicofacial area is subject to a greater risk of disabling complications, such as osteoradionecrosis of the maxillary bones, especially mandibular, xerostomia and hyposcialia, superinfections, painful ulcers and erosions, scarring and trismus, decay of the residual dentition and periodontium [50].

Chemotherapy has been used with various purposes, ranging from palliative treatment to therapeutic treatment and radiotherapy completion process. Cisplatin is still considered as the drug of choicetoday. [51]

1.4 Other variants of oral carcinoma: verrucous carcinoma

In the context of the oral district, a variant of the OSCC that deserves to be considered concerns verrucous carcinoma (OVC), first described and defined as OVC in 1948 by Lauren V. Ackermann (also known as "Ackermann's tumor" or "Ackermann's warty carcinoma") [52].

This type of neoplasm is a low-grade and well-differentiated clinical variant with a warty appearance of OSCC, which from an epidemiological point of view accounts for about 2-12% of all oral carcinomas, in association with a rate of 5-year survival averaged 50% [53].

As for the clinical features, the OVC is a malignant tumor characterized by slow exophytic growth, which generally appears as a warty growth, similar to cauliflower and a mammary surface [54].

In histological terms, this variant mouse exhibits a microscopic characteristic typical of the "push boundary" (both light and electronics) with an invasive local pattern and rare regional and distant metastases [55].

From the etiological point of view, the OVC is associated with multiple factors, taking the form of a pathology of extreme complexity [56].

The main association factors certainly include the consumption of alcohol, smoking, the habit of chewing areca nuts and alterations of the oral microbiota which individually or synergistically contribute to oral carcinogenesis [57].

Other causative factors investigated in the genesis of this neoplasm have included: incongruous prostheses, pre-existing lesions and scars and chronic inflammation associated with autoimmune conditions involving the oral cavity. In some cases, as with OSCC, it can originate in apparently healthy oral mucosal sites or arise in the context of potentially malignant disorders, including oral warty leukoplakia, oral lichen planus, oral submucosal fibrosis (OSF), odontogenic keratocysts [58].

Among the various causative agents of OVC, the pathogenic role of the human papillomavirus (HPV) is widely debated, as shown by numerous works in literature which often lead to very conflicting results. Researchers found the presence of HPV DNA in 12 (48%) out of 25 OVC patients. In particular, the identification of HPV 18 DNA in 40% of OVCs revealed, according to this study, an association between HPV and OVC although the potential etiological and prognostic significance of HPV in OVC deserves further exploration [59]. Conversely, other scholars have argued that the role of HPV may only be occasional as there is

no verified correlation between OVC and HPV. This working group examined the role of HPV in association with OVC development, analyzing 39 OSCC, 8 OVC and 9 normal mucosal samples. In this study, no correlation was established between HPV and OVC because all the tested samples were negative for HPV [60]. Evidently, further studies will be needed to investigate the real role of HPV in the context of this neoplasm in association with the use of highly sensitive molecular biology techniques in order to obtain a greater understanding of the subject.

In reference to the clinical aspects, OVC often originates in the context of the buccal mucosa, in the tongue, lips, gum, alveolar ridge and floor of the mouth [61], showing a predilection for elderly males, especially those over the age of sixty [62]. Its prevalent clinical manifestations are represented by an exophytic development in association with a papillary aspect. Due to its slow growth and the prevailing tendency to local aggression leading to rare regional or distant metastases, OVC has a relatively good prognosis [63]. Based on clinical manifestations

and prognosis, a research group divided OVC into three types: exogenous type, cystoid type, and infiltrative type. The exogenous type of OVC is characterized by exophytic growth, a cauliflower-like warty lesion, and slow tumor growth. However, the other two types of OVC grow rapidly, forming highly thickened keratoses, associated with a poor prognosis compared to the exogenous type of OVC [64].

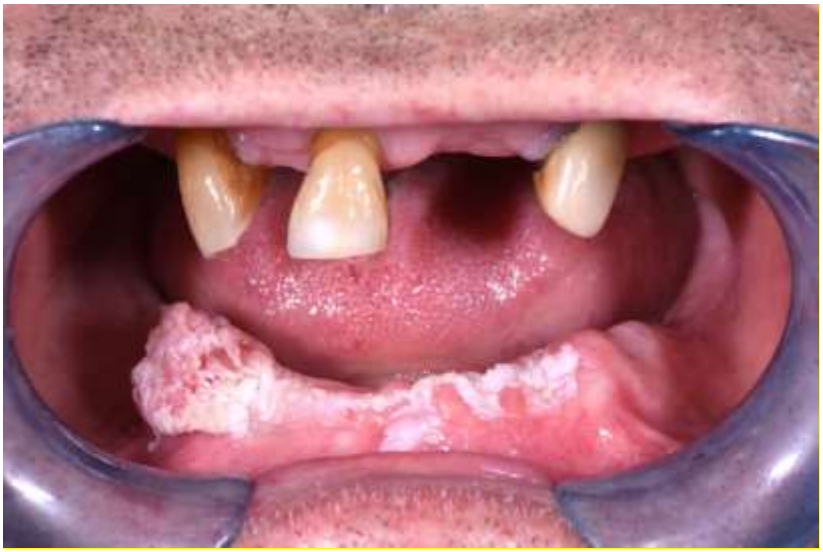


Fig.3. Oral verrucous carcinoma of alveolar ridge.

Regarding histological characteristics, OVC epithelial cells are well differentiated, having a weak cellular

atypia. The squamous epithelium of the OVC shows a highly proliferative papillary appearance and a marked keratosis. The highly proliferative epithelial pegs showswelling with blunt drop-shaped tips. All epithelial pegs are infiltrated into the connective tissue to the same depth, forming the thrust contours [65]. Many lymphocytes and plasma cells are also infiltrated into the connective tissue where cancer cells can degenerate or be ingested by phagocytic cells, resulting in the destruction of the carcinoma cells. Between the squamous epithelium and connective tissue, most of the components of the epithelial basement membrane (BM) of OVC remain intact.

Since its first discovery, few other subsequent works have focused on studying this clinical variant of oral cancer. Although subsequent research into the diagnosis and treatment of OVC was largely initiated at the beginning of this century [66], the progress of research is still far from being completely satisfactory. Even today, in some cases, the differentiation of OVC from OSCC is sometimes difficult by simply observing

the clinical and pathological characteristics, while in someother casesit has been possible to find a biological behavior of OVC similar to OSCC, in terms of tendency to invasion local [67]. The precise identification of this subtype of neoplasm – within the broader group of oral carcinomas - is therefore extremely important, with regard to their different molecular mechanisms and prognosis.

1.5 Long Non-Coding RNA

In recent years, the development of increasingly sophisticated high-throughput sequencing techniques has led to the discovery that only 2% of the human genome is associated with protein-coding genes and that the vast majority of the human genome is actively transcribed as non-coding RNA (ncRNA) (Wright and Bruford, 2011).

Long Non-Coding RNAs (lncRNA) are a ncRNA class longer than 200 nucleotides which - until few years ago - were defined as "junk DNA" [68]. The literature provides

both various classification criteria and different types of definitions about this kind of molecules. Overall, these are obligated transcripts of RNA, that is "non-coding", therefore not associated with transcriptional units encoding known proteins and characterized by a relatively long sequence [69], arbitrarily defined to be greater than 200 nt, to make a distinction with respect to most other groups of small ncRNA transcripts, such as microRNA, tRNA, small nuclear RNA (snRNA), rRNA, small nucleolar RNA (snoRNA). This length has been defined both for practical implications and to provide a classification criterion during the common experimental procedures of RNA separation.

The nucleotide sequence of lncRNA forms its primary structure, while, hydrogen bonds formed by the internal structure of RNA (including the Watson-Crick face, the Hoogsteen face and ribose) build its secondary structure which includes double helices, rings hairpin, bulges and pseudopods.

Furthermore, thanks to their higher structural length, in comparison to other classes of ncRNA, lncRNAs can fold into more complex three-dimensional structures. It

has also been shown that these three-dimensional structures can determine specific interactions of lncRNAs with transcription factors and histones and other chromatin-modifying proteins. Chromatin-modifying proteins can affect the expression level of a broad spectrum of genes [70].

Most lncRNAs are synthesized by RNA polymerase II thanks to an intrinsic greater synthesis activity, while RNAPoli I and III are generally limited to the transcription of shorter RNA transcripts. The primary transcript then undergoes a process of capping at 5', polyadenylation at 3' and splicing to be functional and this process allows the stabilization of the transcripts allowing the preservation of their functional role.

These molecules share many of the biological characteristics of mRNAs and although they have little or no coding potential, some recent studies have suggested a possible ribosomal involvement in producing small polypeptides [71].

Regarding the transcriptional profile, lncRNAs are generally expressed at lower levels than transcripts encoding known proteins. Compared to the latter, their

expression pattern is more specific for the definition both of the developmental stage and of the cell type [72]. Their primary structure and the complex secondary structures of lncRNAs allow them to interact specifically with DNA, RNA and proteins. Since lncRNAs are localized both in the nucleus and in the cytosol, they can act at virtually any level of gene expression by modulating their expression through a series of mechanisms at the epigenetic, transcriptional and post-transcriptional level [73].

LncRNAs can be divided into several functional categories based on the site of action and on the level of gene expression, thus being based on their intrinsic characteristics: (1) according to their position in the context of the genome (intergenic and intronic lncRNA, lncRNA sense and antisense), (2) as a function of their effects on DNA sequences (cis-lncRNA, trans-lncRNA). (3) as a function of their regulatory mechanism (transcriptional, post-transcriptional or other functioning mechanisms), (4) as a function of their possible targeting action [74].

Nuclear lncRNAs can be subdivided into cis-acting RNA, which work in close proximity to their transcription sites, and trans-acting RNAs that operate in distant loci. Both cis and trans-acting lncRNAs can activate or repress transcriptional phenomena through the recruitment of chromatin remodelers and modifiers, thus changing the chromatin status of a particular locus or even of an entire chromosome (Figure 1A) [75].

Nuclear lncRNAs are also able to induce or prevent the transcriptional mechanism, interacting with transcription factors, directly affecting the transcriptional output of a region (Figure 1B). Among these, Enhancer RNA (eRNA), are encoded by extragenic regions and promote the transcription of surrounding genes [76].

Participating also in post-transcriptional coregulation mechanisms at nuclear level, lncRNAs can indirectly interact in various steps with splicing processes or directly with nascent mRNAs to guide them towards particular splicing events. (Figure 1C) [77].

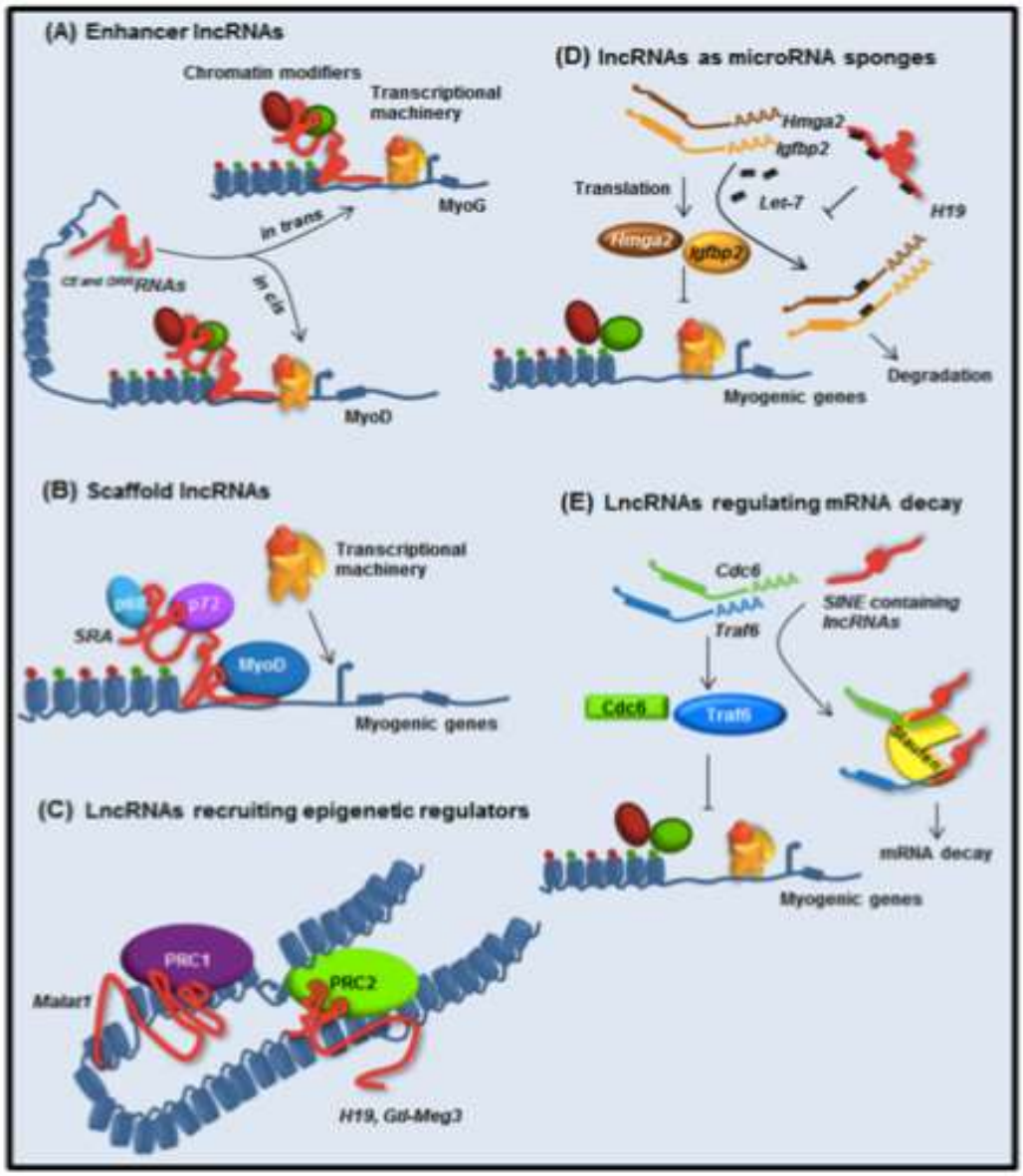
Depending on their regulatory activity and modeling of the subnuclear architecture, it has been observed that some lncRNAs can regulate the chromosomal cycle,

inducing or altering the interactions of the chromosomes themselves (Figure 1D) [78], while some other lncRNAs act as structural scaffolds in the formation and regulation of nuclear compartments under the form of speckles [79] and Polycomb bodies (Figure 1E) [80].

At the cytoplasmic level, lncRNAs influence the outcome of the translation process by acting in various ways. First, they can modulate the speed of the translational process by regulating the size of the polysome on an mRNA molecule (Figure 1F) or by controlling the internal ribosomal entry sites (IRES) [81] and - secondly - they can regulate gene expression by reducing or stimulating mRNA decay (Figure 1G) [82]. A particular class of cytoplasmic lncRNAs, endogenous competitor RNAs (ceRNAs), regulate both translation and degradation rates of mRNAs, acting as molecular sponges for miRNAs, thus modulating the repressive activity of miRNAs on their mRNA targets (Figure 1H) [83].

Overall, lncRNAs exhibit remarkable functional flexibility and tightly regulated expression that give them enormous potential as fine tuners of cellular function and identity. Thanks to their versatility, lncRNAs are able to

control different aspects of cell development, from maintaining stem cells to both engagement and differentiation and we expect their biological role in a wide variety of cell types to be discovered in the near future [84].



Neguembor MV, Jothi M, Gabellini D. Long noncoding RNAs, emerging players in muscle differentiation and disease. *Skelet Muscle*. 2014;4(1):8. Published 2014 Mar 31. doi:10.1186/2044-5040-4-8

Fig.4. Role of LncRNAs in mediation with modifiers of chromatin activity and transcription factors.

1.6 LncRNA and OSCC

In the last decade the role of miRNAs in oral squamous cell carcinomas (OSCC) has been extensively studied. However, the functional role of lncRNAs in such tumors remains unclear.

Due to their abundant presence in the human genome, and thanks to the characteristic of having tissue-specific expression patterns, the functional relevance of lncRNAs has assumed, in recent years, an increasingly greater value both in the study of physiological and pathological states, and in particular in the carcinogenic mechanism.

In fact, these molecules play a fundamental role in gene regulation in relation to cell proliferation, survival, migration, and genomic stability. An alteration in the expression of these molecules has been associated at the beginning, at the progression of cancer, and at the spread on a local and systemic level [85].

In most cases, the presence of an altered expression profile of a given lncRNA is associated with an effect on the cancer phenotype. Such effect leads to an onco-suppressive or oncogenic action, participating in various

cellular processes such as proliferation, differentiation, invasion tumor, and metastases.

For these reasons, numerous studies have focused both on evaluation and comparison of the expression profiles of these non-coding molecules at the level of the normal oral mucosa with respect to the profiles found in frankly tumor tissue, even if the tumor suppressor or oncogenic function cannot be only determined on the basis of differential expression of lncRNAs in cancer. This also depends on predictions based on functional studies [86].

The main works found in the literature that discriminate the expression profile of lncRNA in OSCC, compared with the profiles of the healthy mucosa, have identified numerous transcripts associated with alterations in expression such as: HOTAIR; MALAT1 NEAT-1; HULC; MEG-3; UCA1 [87].

The HOTAIR transcript (Hox antisense intergenic RNA) represents perhaps one of the most studied lncRNA and associated with a wide variety of cancers, including breast, colorectal, nasopharyngeal and liver cancers [88]. HOTAIR was the first lncRNA found to associate

with PRC2 complexes, initiating the subsequent characterization of a large number of RNAs interacting with PRC2, later known as the PRC2 transcriptome. HOTAIR is also able to cooperate with the LSD1 complex and can be regulated by various factors such as miR141, Ago2, c-Myc, TGF-β and small interfering RNAs (siRNAs), modulating various modifications at the epigenetic level [89].

Numerous studies have therefore focused on the evaluation of the expression profile of this LncRNA in the OSCC and almost all of them have discriminated against its overexpression [90],[91].

On the contrary, few studies have taken into consideration the expression of HOTAIR in dysplastic lesions and in any case did not reveal significant differences in expression [92].

Even if these studies are to be considered as preliminary not yet confirmedresults, they may suggest a possible variation towards the overexpression of this transcript contextually to the progression towards a malignant transformation.

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also called nuclear enriched abundant transcript 2 (NEAT2), is an intergenic lncRNA (LINCRNA) that maps to chromosome 11q13. MALAT1 is involved in the proliferation, migration, invasion and apoptosis of tumor cells and in vitro, it has been found that MALAT1 acts as a sponge that downregulates miR-125b and that, conversely, can lead to a significant upregulation of STAT3, thus favoring proliferation cell in colony formation assays.

The expression of this lncRNA has been taken into consideration by numerous studies, which have found a significant overexpression especially with regard to TSCC[93], while no significant differences were found in terms of expression in the OSCC [90]. The only study addressing MALAT1 expression in dysplastic lesions found significant under-expression [92], suggesting that overexpression in OSCC, if demonstrated, could be related to progression from dysplasia to carcinoma.

Other interesting studies have recently found, in a large cohort of patients, a significant overexpression of NEAT1 [94]. Something worth noticing is that other

studies have shown a downregulation of NEAT1 in the presence of dysplasia, suggesting a potential change in its expression during carcinogenesis [92].

The increased expression of lncRNA MEG3 has also been evaluated by other studies, and also in this case it has been consistently found in both OSCC and TSCC [95]. Several studies have then evaluated the expression profile of UCA1 with similar results in cancers of the oral cavity of multiple anatomic sites underlining how the overexpression of this lncRNA represented a significant cost in OSCC [96]. Other authors estimated that UCA1 overexpression was not observable in the presence of dysplasia [92].

Other studies on the expression profile of OSCC-related lncRNAs evaluated FTH1P3 overexpression, HIFCAR overexpression [97], and CCAT2 overexpression [98].

Colon cancer-associated CCAT1 and CCAT2 transcripts were also reported to behave differently in OSCC, finding that CCAT1 was overexpressed in only 27% of OSCC while CCAT2 was significantly overexpressed [98].

PTENp1 was almost absent in OSCC compared to adjacent normal tissues [99], and therefore a significant underexpression found even in the presence of dysplasia, thus suggesting an early involvement of this transcript in tumorigenesis [92].

LncRNA	Location	Behavior	Expression	Target	Reference
AC132217.4	UM-SCC6H and SCC-090 cells	Biomarker	Over-expressed	IGF2	Li X, 2017
CCAT1	OSCC tissues/HIOECs	Biomarker	Over-expressed	miR155-5p, miR490-3p, miR218-5p	Arunkumar G, 2017
FTH1P3	SCC-4, SCC-9, SCC-1, SCC-25, TU-183, SCC-15 cells	Oncogene	Over-expressed	miR-224-5p	Zhang CZ, 2017
MALAT1	Tca8113, SCC-25, CAL-27 and HN5 cells	Biomarker	Over-expressed	miR-125b	Chang SM, 2017
MEG3	OSCC tissues/SCC-15 and CAL-27 cells	Biomarker, Tumorsuppressor	Under-expressed	miR-26a	Yang YT, 2016
NEAT1	HN-4, Tca-8113, UM-SCC-1, SCC-25 and SCCKN cells	Biomarker	Over-expressed	miR-365	Yu X, 2017
HOTAIR	TSCCA, Tca8223, KB and CAL-27 cells	Biomarker, Oncogene	Over-expressed	EZH2 and H3K27me3, MCL-1	Wang X, 2018
UCA1	SCC-15 and CAL-27/Tca8113, CAL-27 and SCC-9 cells	Biomarker, Oncogene	Over-expressed	miR-184	Fang Z, 2017

Tab.1. Main LncRNA in OSCC, function and biological target.

1.7 LncRNA expression and correlations with the anatomical site of origin

Some of the lncRNAs associated with OSCC have shown well-defined roles in cancer development and progression, suggesting that these lncRNAs can be used as novel biomarkers and monitoring tools, as well as potential therapeutic targets in the treatment of OSCC, thanks to their specificity in terms of expression linked to the anatomical site of onset of the neoplasm.
In fact, by comparing the expression levels within the larger chapter of squamous cell carcinomas of the head and neck district (HNSCC) it is possible to identify the presence of transcripts expressed in a different way between different types of cancer of different anatomical sites, thus suggesting that many lncRNA can serve as tissue-specific biomarkers [100].In this sense, for example, a characteristic overexpression of HOTAIR was evaluated in squamous cell carcinomas of the oral cavity (OSCC) that originated in the various oral anatomical sub-sites, but not at the lingual level. On the contrary, a reduced expression of the lncRNA GAS5

was demonstrated only in lingual squamous cell carcinomas (TSCC), which are not detectable in other tumors from other areas of the oral cavity.

None of the above lncRNAs have been associated with a transcriptional alteration in the laryngeal SCC [86].

In a further study a different expression of lncRNA H19, AP5M1 and MALAT1 was evaluated in squamous cell carcinomas of the oral cavity OSCC, when compared with the levels of expression found in squamous cell carcinomas of the head and neck district (HNSCC), allowing to define a characteristic tissue-specific expression of lncRNAs and the fact that HNSCC fits into a heterogeneous group of tumors with different epigenetic alterations [90].

The non-coding transcript CCAT1 has been found being more OSCC expressed in heavy smokers or in tobacco chewing habituated patients. Furthermore, its greater expression was mainly documented in carcinomas originating in the buccal mucosa, in comparison to other oral anatomical sites. In this sense, the differential expression of CCAT1 found could be influenced both by

exposure and risk factors and by the anatomical site of onset of the neoplasm [101]

1.8 LncRNA expression as a function of tumor characteristics and TNM staging

More recently, a correlation was sought between the characteristics of tumor development in terms of size and locoregional involvement and lymph node metastatic spread with a possible aberrant expression of the main lncRNAs of oncological interest.

Following this line of research, some studies reported that in the presence of nodal involvement the expression of HOTAIR, NEAT1 and UCA1 was significantly upregulated, while MEG3 was downregulated [102].

HOTAIR has been extensively investigated in numerous papers. The majority of them have supported a significant link between overexpression of this transcript and the presence of nodal metastases [102].

Such a trend associated with metastases has been related to increased tumor stem cell and metastasis due

to HOTAIR overexpression in oral cancer stem cells [91].

One author hypothesized that UCA1 overexpression promotes the metastatic but non-proliferative capacity of TSCC cells [103].

On the contrary, the link between lymph node involvement and the under-expression of MEG3 is not associated with a uniformity in the result [90].

Three studies reported conflicting results in MALAT1 evaluation, overall suggesting that a significant overexpression in the presence of nodal involvement could only be found in TSCC 77 but further validation is required [90], [102].

Most TNM stage studies have compared lesions with stage I-II versus lesions with stage III-IV disease. When both TNM stage and nodal involvement were evaluated, some transcripts (CCAT2, EGFR, HOTTIP, PTENp1) appeared to be associated with tumor size rather than nodal involvement [99], [104], while others appeared to be related to nodal involvement but not tumor size (CEBPA-AS1, HAS2-AS1, MBL2-4: 3, TUG1, UCA1) [103], [105], [106].

Finally, it should be noted that among the various evaluated transcripts in the literature regarding the possible association with both nodal involvement and tumor size, only LINC00152 [107], lnc-p23154 [108], and NKILA [109], showed a common significant alteration of the expression profile: over-expression in LINC00152 and lnc-p23154, under-expression in NKILA.

1.9 LncRNA and prognosis

Some studies in the literature focused on the correlation between lncRNA expression and the 5-year prognosis. Some of these studies have assessed the survival rate in the absence of relapse. In most cases, when a significant link between lncRNA expression and prognosis was assessed, this expression was also related to the presence of nodal metastatic involvement.
In a first study, it was revealed that H19 overexpression was significantly correlated with a poorer prognosis [110].
The estimated survival analysis was then evaluated in a cohort of OSCC patients and assessed that higher

HOTAIR expression was associated with poor survival [91].

HOTAIR was probably the most investigated transcript. Its prognostic role has been evaluated in multiple independent cohorts of OSCC patients. Despite the presence of conflicting results derived from studies exploring the association between HOTAIR expression and nodal involvement or tumor classification, almost all of them have found a negative impact of HOTAIR overexpression together with the association of this expression profile with a poorer prognosis [85], [111].

2. EXPERIMENTAL PART

2.1 Rationale for the study

Several functional analyzes made on various cell lines and on animal models have shown that lncRNAs can be configured as key genomic regulators in a wide variety of biological pathways, including differentiation and development [113]. Based on these considerations, many studies have confirmed that a large number of lncRNAs are associated with an alteration of the expression profiles in various tumor types of different anatomical districts compared to the corresponding normal tissues. These lncRNAs are considered as key agents in the development, progression and possibly in the treatment of cancer [114].

Growing evidence has therefore suggested that lncRNAs may be configured as useful biomarkers in the diagnosis and prognosis of cancer with respect to protein-coding genes. This is possible thanks to lncRNAs specificity expression related to cell type, tissue of origin, and disease status, in relation to genes

encoding proteins. Moreover, the study of the expression profile could represent an indicator of the neoplastic state [115], [116].

The aim of the work is - in this sense - to carry out an observational retrospective study through the evaluation of histological and molecular biology analysis about the possible correlation between the expression profile of a lncRNA series in tissues frankly associated with metastatic and non-metastatic OSCC or in the OVC compared to control tissues, and compared to the general neoplastic behavior, in terms of local aggression, capacity for metastasis, preferential site of origin, and correlated prognosis.

Through a careful analysis of the literature we tried to clarify the role played by the most cited lncRNAs, which are characteristics of different phases of the pathogenetic process of squamous cell carcinoma, so as to identify reliable biomarkers to better characterize the neoplastic staging.

At the moment the staging is exclusively clinical and radiographic according to the TNM system, which does not often allow an objective forecast of the local or

systemic behavior of the tumor and does not provide any information, nor on the prognosis nor on the usefulness or otherwise of a certain surgical or radio-chemotherapeutic approach.

Despite public awareness campaigns carried out by scientific societies and government bodies in recent decades, the survival rate has remained unchanged, being around 50%. Therefore, refining the clinical study of this neoplasm using biomarkers able to predict local aggressive behavior, the ability to metastasize, the possibility of relapse, and the response to treatments it is considered as appropriate.

The usefulness of the study is to provide planning tools to more personalized approaches to the patient, trying to be more incisive and targeted down to the molecular level in the eradication of the disease in affected patients or in a more conscious management of the quality of life in case of no longer surgically removable lesions.

2.2 Definition of the different research objectives

• To carry out an analysis of the scientific literature aimed at identifying the most significant lncRNAs that may be associated with the different histological, clinical, and possibly prognostic aspects of squamous cell carcinoma of the oral cavity.

• To identify which lncRNAs are useful for a possible classification of neoplastic behavior in terms of local aggression, lymph node metastatic spread, probability of recurrence.

• To collect clinical documentation and histological samples in paraffin from patients - under treatment at the Circolo Hospital - diagnosed with oral squamous cell carcinoma or verrucous carcinoma and to verify their five-years survival rate.

• To perform molecular biology studies using real-time PRC to compare the differential expression profiles of 84 lncRNA among samples of *non-metastatic oral*

squamous cell carcinomas (nmOSCC), *metastatic oral squamous cell carcinomas* (mOSCC), and oral*verrucous carcinomas* (OVC), in relation to the control of samples of healthy mucosa, with the aim of evaluating any differences.

• To proceed with the statistical study of the data through a univariate analysis for the evaluation of variations in the expression profile of *neoplastic tissues* (K) compared to *healthy tissues* (CNT) for each of the three subgroups nmOSCC, mOSCC, OVC.

• Perform a multivariate analysis to discriminate the presence of a possible differential expression profile among the samples of the three subgroups (nmOSCC, mOSCC, OVC), through correlating the histological type of the identified neoplasms with the one of lncRNA expression.

3. MATERIALS AND METHODS

3.1 Definition of population and examination groups

The population to which the study is addressed is made up of patients with carcinoma of the oral cavity whose diagnosis was identified at the Oral Medicine clinic of the SC of Odontostomatology of the Circolo Hospital from 2008 to 2013 for a total of 9 patients, divided as follow: 3 non-metastatic oral squamous cell carcinomas nmOSCC, 3 metastatic oral squamous cell carcinomas mOSCC and 3 verrucous carcinomasOVC. The choice of the time period is dictated by the need to verify the survival rates, absence of disease or relapse after 5 years.

Further subdivisions of the study sample were then conducted in relation to the site of onset, the habit of smoking and alcohol or in relation to the absence of risk factors, survival or death, etc. All these aspects are influenced by the dysregulation of lncRNA.

In order to find the documentation stored in the medical record was therefore examined to find the following information on patients: sex, age, presence of risk factors, topographical site of the neoplasm, number of histological examinations carried out by the Pathological Anatomy Service of the Circolo Hospital, surgical treatment, chemoradiotherapy treatment, presence of cytopathic changes from HPV in the context of histological examination, relapse or absence of disease at 5 years, survival or exitus at 5 years, presence or absence of cervico-facial lymph node metastases, presence or absence of distant metastases, degree of neoplasm, stage of neoplasm at the time of diagnosis.

An informed consent form was administered to those patients still attending our department for follow-up visits and to those who were not attending our clinic anymore but were available over the phone. The current study was authorized by the Institutional Ethics Committee of Insubria.

	GENDER	AGE'	SMOKE	TYPE	LOCALIZATION	STAGE	GRADE	5-YEAR SURV
1	M	73	N/A	nmOSCC	TONGUE	2	2	0
2	F	74	N/A	OVC	FLOOR	1	1	1
3	F	55	EX	mOSCC	FLOOR-VENTRE TON.	3	2	0
4	F	87	NO	mOSCC	FLOOR	4	2	1
5	F	80	NO	nmOSCC	TONGUE	1	2	0
6	F	52	EX	nmOSCC	TONGUE	1	2	0
7	F	88	EX	mOSCC	TONGUE-FLOOT	4	2	0
8	F	72	(5/die)	OVC	GUM-LIP INF	3	1	1
9	M	85	(5/die)	OVC	CHEEK	2	1	1

Tab.2.Differentiation and the groups under consideration based on the type of neoplasm, site of onset and lesional and prognostic characteristics.

Once the clinical data collection has been carried out and the names of the patients eligible for the retrospective study and the histopathological report number indicated in the file have both been accurately identified, the Pathological Anatomy Service of the Circolo Hospital was requested to recover the corresponding stained slides in hematoxylin eosin.

3.2 Inclusion and exclusion criteria

The study includes patients whose diagnostic and therapeutic path was carried out within the Circolo Hospital, so to avoid dispersion of data or difficulties in finding the missing information for the study.

The samples stored in paraffin had to have sufficient material to be reused in order to proceed with molecular biology studies, while leaving the material in storage in the Pathological Anatomy archive.

The normal control mucosa, adjacent to the neoplastic tissue and sampled at the time of tumor excision from the apparently normal tissue not connected to the excised cancer sample, had to be spaced at least 15mm from the latter, to exclude - at least partially - the possibility of a bias related to field carcinization.

Patients who have undergone a diagnostic or therapeutic journey in facilities outside the Circolo Hospital were excluded from the data collection.

Samples that are insufficient for in-depth analysis or whose residual material would become insufficient for archival storage (exhaustion) were taken into consideration.

Patients diagnosed with metastatic squamous cell carcinoma from other anatomical sites or other oral carcinomas other than those mentioned above are excluded.

3.3 Methods of analysis in the laboratory

The analysis on the expression profile of 84 lncRNA was conducted on histological samples from patients, which are stored in the Pathological Anatomy Service of the Circolo Hospital, using RT2 lncRNA PCR Array Kit for Human Cancer Pathway, Qiagen.

The paraffin samples were processed using a mechanical sampling phase, thanks to an extraction template, which allowed the collection of both cancerous

tissue and considered healthy tissue, which has been used as control, being at least 15mm away from the neoplastic tissue.

In particular, each sample from each patient has been cut in at least 4 sections, each section being10 μm thick, in association with a standardized extraction surface of 250 mm2.

All of these samples were then subjected to an elimination step of purified paraffin with 1ml xylene.

Next step has then involved the extraction of total RNA and ncRNAs, throughout the use of the miRNeasy FFPE Kit, Qiagen.

The quantification of the total nucleic acids and therefore of the total RNA was then conducted using a nanodrop.

Before proceeding to the reverse transcription phaseit was decided to use a standardized quantity of RNA - equal to 110 nanograms - for each sample.

The genomic DNA was then eliminated in order to remove any residual contamination in the RNA samples before reverse transcription, thus eliminating false positive signals.

The RNA was then subjected to the retro-transcriptional phase and converted to complementary DNA (cDNA) to increase its stability using the RT 2 First Strand kit for the synthesis of Qiagen cDNA.

Subsequently, the pre-amplification phase was conducted using specific primers for the selection of the genes of interest with the RT2 lncRNA PreAMP Primer kit, Qiagen.

A selective elimination of residual primers was executed before carrying out the PCR.

Subsequent real-time PCR molecular biology investigations were then carried out using specific array assays, which were customized in Syber Green for the definition of the expression profile of 84 lncRNAs. These lncRNAs are known to be differentially expressed in numerous neoplasms of various anatomical districts, compared to normal tissue, using the RT 2 lncRNA PCR kit together with the RT2 SYBR Green Mastermix for qPCR, in association with a Quant Studio 3 Real Time digital instrument, Thermofisher Scientific.

Inside the plate there were also: control of reverse transcription, control of PCR, and control of genomic DNA contamination.

During the comparison between control oral mucosa and neoplastic tissue, the following data have been studied and quantified: the statistically significant levels of modification in the expression profile of the lncRNA transcripts detectable in neoplastic tissues, compared to control ones, or in healthy mucosa. It has also been defined the presence of a“alteration of the expression profile of a lncRNA based on the expression related to the transcript normalized with the expression of a standard reference gene called housekeeping, coamplified together with the target of interest, in particular β-actin”.

3.4 Statistic analysis of the expression profile

A relative quantification of the changes in expression level in multiple samples was conducted, related to an internal control, i.e. a reference gene, coamplified together with the target of interest.

Position	UniGene	GenBank	Symbol	Description
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H04	N/A	NR_001445	RN7SK	RNA, 7SK small nuclear
H05	N/A	NR_002907	SNORA73A	Small nucleolar RNA, H/ACA box 73A
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Tab.3. Reference gene coamplified together with the target of interest.

The preparation of the data available, related to the nine selected cases, provided for the subdivision into three study groups, have been translated into 3 data folders (Classic metastatic, Classic non metastatic and Verrucous), in association with a file in.tsv format

containing the list of 84 lncRNAs investigated with descriptions of each well, plus the control wells.
Each data folder contains the sheets defined as "results" of the original data in xlsx format, with the following encoding:

After patient IDs and tissue IDs have been defined, they have been used for reading and classifying data for analysis. The study of the various amplification cycles envisaged that starting from the line containing the string defined "Well" and for 96 consecutive lines, the threshold cycle values (Ct) for healthy tissue samples (CNT) and associated samples were read cancer (K) referred to tissue ID.

The relative expression of the analyzed samples was then calculated by obtainingthe values of ∆C_t in the first analysis using the below equation:

$$\Delta C_t = C_{t,housekeeping} - C_{t,gene}$$ This value is equal to:

$$-\log_2 \frac{gene}{housekeeping}$$

For values of ∆C_t> 0 the increase in expression is borne by (K)

For values of ∆C_t<0 the increase in expression is borne by (CNT)

The term ∆∆C_t was then calculated, for the quantification of the relative variations of gene expression in tumor tissue compared to healthy tissue in the same subject:

$$\Delta\Delta C_t = \Delta C_{t,CNT} - \Delta C_{t,K} = -\log_2 \frac{K}{CNT}.$$

The data related to the relative expression in the three subgroups, with respect to the healthy tissue of the same subject, have been evaluated by conducting an univariate analysis, that is, by individually evaluating the expression of individual lncRNAs in the neoplastic tissues and in the control tissues. A two-tailed parametric type test (Student's t) and a sample have been conducted for each examined subgroup or folder, in order to establish the probability that the mean variation is zero. This variation has been considered to be significantly non-zero for genes exhibiting $p \leq 0.05$ In the following phase, through a multivariate analysis, it has been decided to evaluate the trends in terms of lncRNA expression, directly comparing the three Classic metastatic, Classic non-metastatic, and Verrucosal groups, to evaluate a possible distancing and differentiation. The matrix containing the $\Delta\Delta C\_t$ of all patients has been scaled and centered in order to avoid bias due to the differences between expression variations in the different single lncRNAs. A classifier has been then obtained by means of sPLS-DA (sparse Partial Least Squares Discriminant Analysis).

4. RESULTS

Classic Metastatic (mOSCC)

Position	UniGene	GenBank	Symbol	Description	log2fold	stdev	p-value
41	D05	Hs.433151	NR_024065	LINC00312	Non-proteincoding RNA 312	1.768	0,02
56	E08	N/A	NR_102270	NAMA	Non-protein coding RNA, associated with MAP kinase pathway and growth arrest	1.054	0,02
65	F05	N/A	ENST00000519282	PRNCR1	Prostate cancerassociated non-coding RNA 1	4.14	0,013
80	G08	N/A	ENST00000466156	TUSC7	Tumorsuppressor candidate 7 (non-proteincoding)	2.738	0,012
83	G11	Hs.529901	NR_001564	XIST X	(inactive)-specific transcript (non-protein coding)	1.298	0,027

Tab.4. Description of lncRNAs with p <0.005 associated with alteration of the expression profile in mOSCC, compared to healthy tissues of the same patient.

Classic Non Metastatic (nmOSCC)

Position	UniGene	GenBank	Symbol	Description	log2fold	stdev	p-value
22	B10	Hs.122718	NR_002785	GNAS-AS1	GNAS antisense RNA 1	3.621	0,022
29	C05	Hs.197076	NR_003716	HOTAIR	Hoxtranscriptantisense RNA (non-proteincoding)	2.285	0,048
31	C07	N/A	ENST00000421733	HOTTIP	HOXA distal transcript antisense RNA	3.237	0,027
45	D09	N/A	ENST00000594200	LINC01233	Long intergenic non-proteincoding RNA 1233	3.075	0,014
47	D11	N/A	GU228577	LSINCT5	Long stress-induced non-coding transcript 5	3.061	0,023
62	F02	N/A	ENST00000519319	PCAT1	Prostate cancerassociatedtranscript 1 (non-proteincoding)	8.277	0,043
65	F05	N/A	ENST00000519282	PRNCR1	Prostate cancerassociated non-coding RNA 1	7.758	0,027
74	G02	N/A	AK024556	SPRY4-IT1	SPRY4 intronic transcript 1 (non-protein coding)	3.031	0.02
76	G04	N/A	ENST00000363312	TERC	Telomerase RNA component	1.408	0,046

Tab.5. Description of the lncRNAs with p <0.005 associated with alteration of the expression profile in nmOSCC, compared to healthy tissues of the same patient.

Verrucous carcinoma (OVC)

Position	UniGene	GenBank	Symbol	Description	log2fold	stdev	p-value
4	A04	N/A	ENST00000601203	AIRN	Antisense of IGF2R non-protein coding RNA	3.481	0,009
19	B07	N/A	ENST00000419650	GACAT1	Gastric cancer associated transcript 1 (non-protein coding)	-597	0,001
26	C02	N/A	ENST00000557544	HIF1A-AS1	HIF1A antisense RNA 1 [Source:HGNC Symbol;Acc:43014]	3.753	0,011

Tab.6. Description of the lncRNAs with p <0.005 associated with alteration of the expression profile in the OVC, compared to healthy tissues of the same patient.

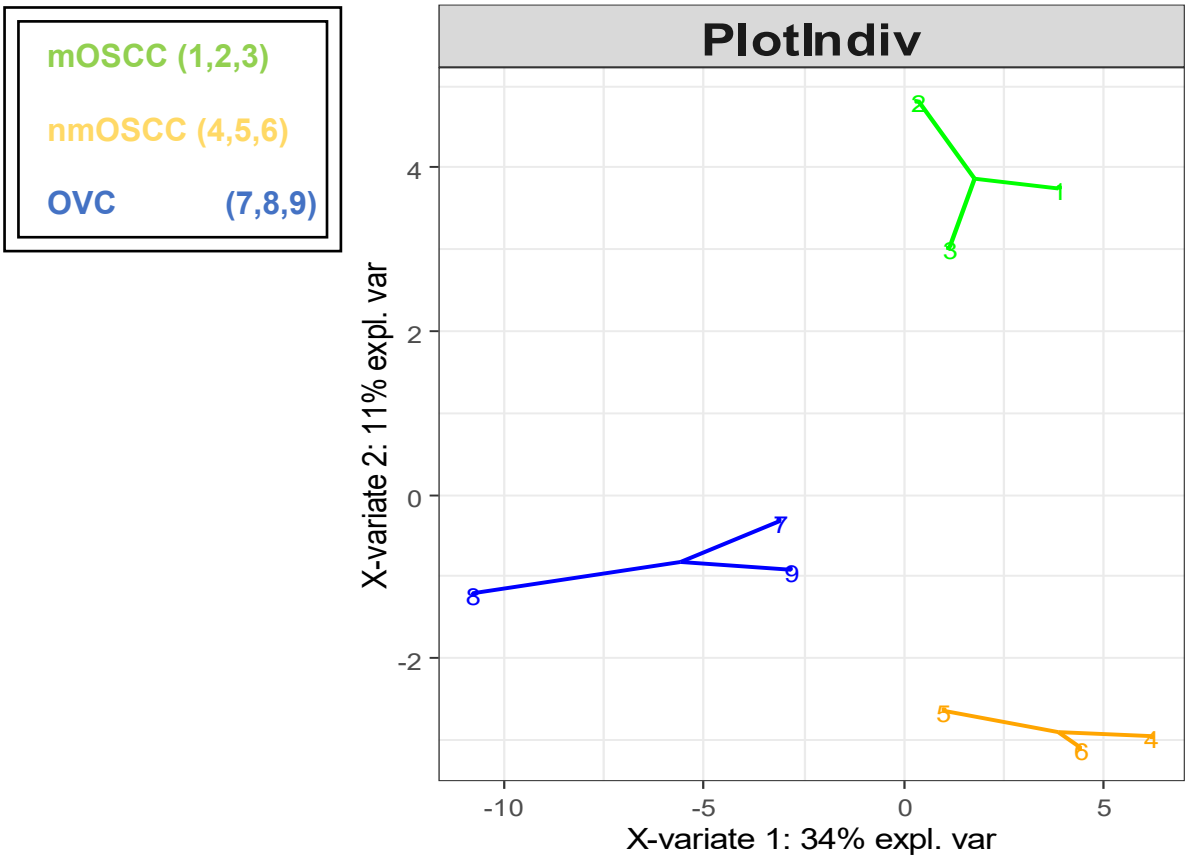
From the results summarized in the tables, it is possible to highlight a significant considerably alteration of the expression profile of 5 lncRNA in the neoplastic tissue associated with the mOSCC sample, and in particular LINC00312, NAMA, PRNCR1, TUSC7, XIST X compared to control tissues.

As regards the nmOSCC group, an alteration of the expression profile of: GNAS-AS1, HOTAIR, HOTTIP, LINC01233, LSINCT5, PCAT1, PRNCR1, SPRY4-IT1, TERC was found.

Regarding the last OVC group, the following have been identified as regulated: AIRN, GACAT1, HIF1A-AS1.

In most cases, the analysis showed a profile tending to overexpression.The use of multivariate analysis in

comparing the pattern of expression among groups: mOSCC, nmOSCC, OVC, associated with the use of both one and two components, found a good differentiation among the three groups in terms of expression.



Graf.1. ROC curves of the three samples in multivariate one and two component analysis. You can see the evident separation of the expression profile in the one-component analysis and even more in the two-component one.

The two-component analysis made it possible to infer a ranking of distancing and maximization of variance, obtaining a good separation of the groups, with 45% of the total variance being described by the two main components.

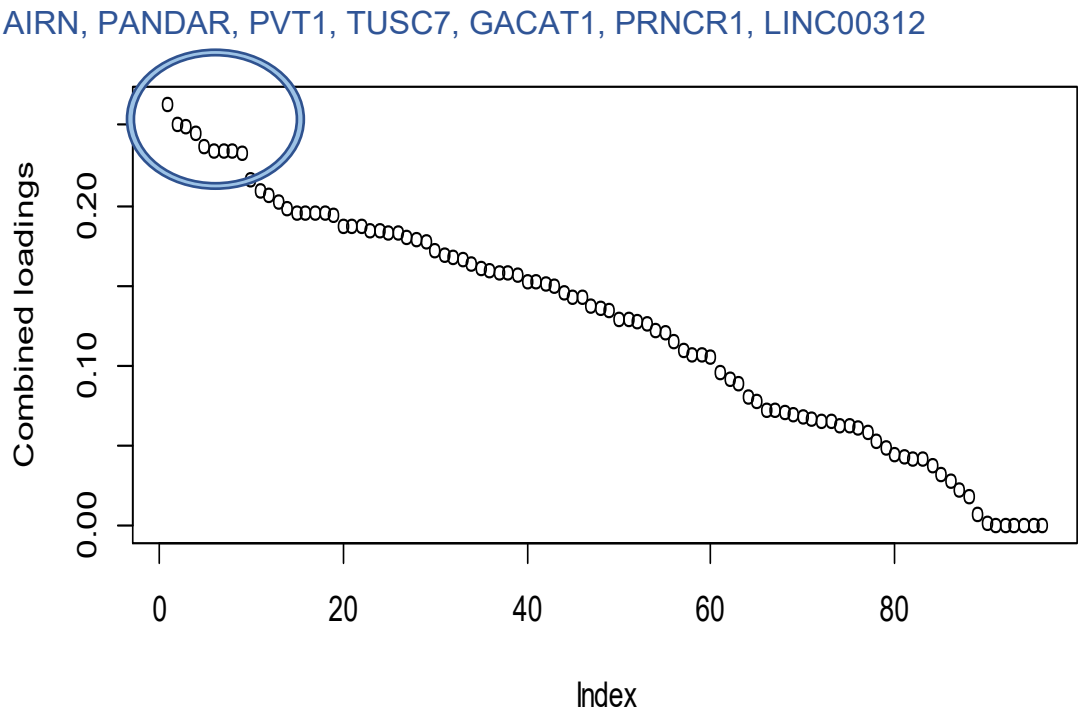
Therefore, the presence of this spacing in terms of variance has certainly shown significantly different profiles among the three groups, as figured out by the area under the ROC curve (AUC), which is always equal to 1 in the 2-component analysis.

	AUC 1	p-value	AUC 2	p-value
CLASSIC METASTATIC vs Other(s)	0.61	0.606	1	0.020
CLASSIC NON METASTATIC vs Other(s)	0.89	0.071	1	0.020
VERRUCOSO vs Other(s)	1	0.020	1	0.020

Tab.7. Values of the area under the ROC curve (AUC) in one and two-component analysis

The subsequent analysis of the combined loadings for the two main components ($\sqrt{PC1^2 + PC2^2}$) shows that we can focus on the 10 major combined loadings,

allowing us to define which is the most responsible lncRNA for the separation between the three groups.



Graf.2. Graphic representation of the most represented combined loadings: AIRN, PANDAR, PVT1, TUSC7, GACAT1, PRNCR1, LINC00312

The following table lists the lncRNAs which show the 10 highest combined loadings and therefore the most associated in differentiating the expression profiles of these three types of oral neoplasms.

Position	UniGene	GenBank	Symbol	Description	Combined loadings
A04	N/A	ENST00000601203	AIRN	Antisense of IGF2R non-protein coding RNA	0.263
E12	N/A	NR_109836	PANDAR	Promoter of CDKN1A antisense DNA damage activated RNA	0.250
F09	Hs.675281	NR_003367	PVT1	Pvt1 oncogene (non-proteincoding)	0.249
G08	N/A	ENST00000466156	TUSC7	Tumor suppressor candidate 7 (non-protein coding)	0.245
B07	N/A	ENST00000419650	GACAT1	Gastric cancer associated transcript 1 (non-protein coding)	0.237
F05	N/A	ENST00000519282	PRNCR1	Prostate cancer associated non-coding RNA 1	0.234
D05	Hs.433151	NR_024065	LINC00312	Non-protein coding RNA 312	0.233
C01	N/A	NR_045680	HEIH	Hepatocellular carcinoma up-regulated EZH2-associated long non-coding RNA	0.233
E10	N/A	NR_028272	NEAT1	Nuclear paraspeckle assembly transcript 1 (non-protein coding)	0.232
F12	N/A	ENST00000455390	RPS6KA2-AS1	RPS6KA2 antisense RNA 1 [Source:HGNC Symbol;Acc:40511]	0.215

Tab.8. LncRNAs showing the 10 highest combined loadings.

5. DISCUSSION

OSCC is the second most common malignancy in the head and neck, alongside with nasopharyngeal cancer. Depending on anatomical causes, related to high vascularity, lymphatic distribution and frequent movement, OSCC is characterized by a high proliferation and early metastatic tendencies. Even advanced surgical treatment combined with neoadjuvant chemotherapy is associated with a 5-year survival rate that is still below than 50%. Therefore, it is deemed necessary to explore more effective therapeutic targets for this type of neoplasm.

Recently, together with the most well-known biological and molecular factors associated with the OSCC genesis process, it has been widely demonstrated that even complex genetic, transcriptional, and epigenetic alterations along with their interaction can actually contribute to the tumorigenesis of OSCC [117].

Traditional diagnostic methods, such as biopsy, radiographic evaluations, and chest CT are partially inadequate to get an objective evaluation of the profile

and intrinsic behavior of the tumor, as well as its evolution. For this reasonit has been decided to move towards the search for specific and sensitive biomarkers for a deeper diagnosis of OSCC.

Numerous alterations in molecular profiles have been observed in various human cancers compared to corresponding normal tissues and they have been then used as novel markers for cancer diagnosis and prognosis [118].

LncRNAs are recently emerging as a new promising biomarker for cancer diagnosis and prognosis thanks to the increasing efforts on the characterization of these molecules in various human cancers. Recent studies have also observed many alterations in the expression profile of lncRNA with implications in an oncogenic or oncosuppressive sense at the tumor level.

Despite these previous investigations, the expression pattern and diagnostic role of lncRNA in OSCC remain unclear.

For these reasons, in this study we investigated the expression profile of 84 lncRNA in oral cancer tissue samples (K), with both different histological features and

different behaviors, compared to the healthy control mucosa (CNT), by means of PCR RT molecular biology techniques. We then analyzed the data by univariate and multivariate analysis. The analysis of the lncRNA expression files in the metastatic, non-metastatic and OVC, OSCC samples and of the control samples, has allowed to detect characteristics and significant alterations between the groups under examination compared to the control tissue, as a first step towards identification of diagnostic biomarkers of lncRNA.

A total of 17 lncRNAs were determined as lncRNAs expressed differentially for p values <0.05, within the three analyzed groups of neoplastic tissue, compared to control tissues.

The following have been identified for the mOSCC group: LINC00312, NAMA, PRNCR1, TUSC7, XIST X, RN7SK.

Regarding the nmOSCC group, a statistically significant alteration of the expression profile of: GNAS-AS1, HOTAIR, HOTTIP, LINC01233, LSINCT5, PCAT1, PRNCR1, SPRY4-IT1, TERC was found.

While regarding the last OVC group, the following were identified: AIRN, GACAT1, HIF1A-AS.

We therefore tried to define which - among these and based on their expression profile - possible diagnostic biomarkers with possible tumor suppressor or oncogenic roles, in relation to the data relating to the stage of the disease, subgroup of the sample site of onset.

After a careful search in the literature, we found that several lncRNAs identified in our study were reported to be involved in models of carcinogenesis and tumor evolution characteristic of other malignant neoplasms of other anatomical districts.

In the group of mOSCC samples, which were associated with the presence of metastases to the locoregional lymph nodes, an overexpression of LINC00312 (Non-protein coding RNA 312) (p 0.002) was measured compared to the control tissue, which has also been found in studies relating to adenopulmonary carcinoma (ADC). In that anatomical district the expression level of LINC00312 was measured in 124 ADC-coupled tumor tissues and in adjacent non-tumor lung tissues using qRT-PCR. In this case an overexpression of LINC00312

has also been found in patients with ADC metastases compared to patients without metastases (p <0.0001). Clinicopathological analysis revealed that high LINC00312 expression was associated with lymph node metastases (p <0.001), distant metastasis (p <0.001), tumor node metastasis stage (TNM) [119].

Other research has revealed that the overexpression of LINC00312 inhibits proliferation by arresting the progression of the cell cycle from G1 to S phase, however, increasing cell motility and invasion, both in vitro and in vivo via the H-ras / pc pathways. -Raf and JNK2 / AP-1 / MMP1 in cells from nasopharyngeal carcinoma (NPC) samples [120].

An alteration of the NAMA lncRNA expression profile has been evaluated, (p 0.002) also found in other studies, in particular with regard to thyroid neoplastic tissues of papillary carcinoma, more associated with aggressive behaviors [121].

PRNCR1 found on 8q24 has been associated with alterations in its expression profile in aggressive forms of breast cancer, withsimilar overexpression levels to those found in the mOSCC group (P 0.013). A study has

recently conducted, also in this case, an analysis by quantitative reaction in real time of the polymerase chain (RT-qPCR) used to measure the expression levels of selected lncRNAs in tumor tissues obtained from 50 tumor patients breast (BC) versus normal adjacent tissues (NAT) and 50 clinically healthy normal tissues. From the results of this analysis it was found that, given the increased expression of PRNCR1, this could act as an oncogene in BC and a greater expression of this lncRNA was in general associated with worse outcomes in BC women [122].

Growing evidence has indicated that TUSC7 lncRNA may be configured as a novel cancer-suppressing gene in various cancers. However, it is urgent to clarify the function of TUSC7 in oral carcinoma, since, in contrast to most other studies investigating this lncRNA, we found a high level of expression compared to healthy tissue (p 0.012). In most of the research, TUSC7 has instead been evaluated as significantly decreased in the tissues and cell lines of various types of cancer. Furthermore, a study evaluated a correlation between low TUSC7 expression with advanced clinical grades

and poorer overall survival in pancreatic carcinomas. TUSC7 would repress cell proliferation, migration, invasion, epithelial-mesenchymal transition and stemness while facilitating cellular apoptosis of pancreatic carcinoma cells [123].

XIST X lncRNA expression has been associated with altered profiles - using quantitative real-time PCR (qRT-PCR) assays - in numerous studies investigating CRC colorectal cancer cell lines. Among all the works that have been carried out, one among all analyzed 196 clinical samples, evaluating the possible correlations between XIST expression and the clinical-pathological characteristics of the CRC. XIST lncRNA, as in our mOSCC unit (p 0.027), was measured as upregulated in cell lines and CRC tissues ($p < 0.05$) compared to control tissues. Statistical analysis found that elevated XIST expression was correlated with larger tumor size, lymph node metastases, and higher staging. Furthermore, according to this study, an increased expression of XIST could predict poor progression-free survival (PFS) and poor overall survival (OS) of patients with CRC [124].

In reference to the nmOSCC group, therefore not associated with lymph node metastases and with theoretically more favorable clinical characteristics, other lncRNAs emerged with a very well differentiated overall expression pattern compared to the mOSCC and OVC groups, as highlighted by the two-component multivariate analysis. The obtained results, in terms of expression profile, have sometimes differed from those found in the literature, probably due to the fact that all the samples belonging to the group were early neoplastic stages and all with lingual onset.

With reference to this group, for example, a differential expression of GNAS-AS1 in neoplastic tissue compared to control tissue (p 0.022) was evaluated in our study, which has also been found in works on non-small cell lung cancer (NSCLC), where its overexpression was correlated with both the promotion of M2 polarization of macrophages and the progression of neoplastic cells. In the NSCLC clinical specimens investigated by this research, the expression level of GNAS-AS1 was significantly increased in tumor tissues compared to adjacent normal tissues, as found in our sample for

OSCC. The hyperexperience for this neoplasm was also indicative of lymph node metastases, moreover GNAS-AS1 expression was higher than in non-metastatic cases [125].

Long non-coding HOX transcript antisense RNA (HOTAIR lncRNA) is overexpressed in many types of human cancers and has frequently been related to clinical stage and lymph node metastasis in oral squamous cell carcinoma (OSCC).

Overexpression of HOTAIR can promote carcinogenesis and metastasis in many cancers, including breast, colorectal, and ovarian cancers. The proliferation and invasion capacity of tumor cells can be effectively suppressed by voluntary suppression of this drawback [126].

It has been widely reported that HOTAIR is found to be significantly upregulated in primary breast cancer and associated with metastases, compared to normal control breast tissues. Indeed, some studies have proposed that HOTAIR promotes the progression and metastasis of breast cancer cells by combining agRNA (siRNA antigen) to silence the metastatic inhibition gene [127].

HOTAIR's RNA interference would not only reduce the invasion and metastasis of liver cancer cells but, according to some studies, it would also participate in the development and promotion of cisplatin and doxorubicin susceptibility conditions [128]. Recent studies have also shown that HOTAIR inhibits intercellular adhesion proteins (including JAM2, PCDH10 and PCDHB5) and can promote pro-metastatic genes (such as SNAIL and LAMB3) in breast cancer.

HOTAIR expression has also increased in oral squamous cell carcinoma (OSCC) and has been associated with metastases, stages and histological differentiation. Furthermore, HOTAIR overexpression has often been reported to have poor prognosis in patients with OSCC.

The relative expression of HOTAIR is correlated with tumor size and clinical stage. Furthermore, a significant negative correlation was determined between HOTAIR and E-cadherin expression levels in OSCC tissues and cells: HOTAIR would be involved in the regulation of E-cadherin by binding to EZH2 and H3K27me3 on the E-cadherin gene promoter. These results suggest that

HOTAIR expression could be one of the critical targets in progression and metastasis, as well as an indicator of poor survival in OSCC [85]. For our study, the presence of HOTAIR overexpression, which is prevalent in the group of carcinomas not associated with metastases, could be related to the fact that all the neoplasms of this group were in the early stages and perhaps in the prodromal stages to the subsequent lymph node spread. Another explanation for the overexpression of this lncRNA within the group of non-metastatic carcinomas nmOSCC, could lie in the fact that all the tumors in the sample are lingual carcinomas. However, in line with what has been reported in the literature, the overexpression of HOTAIR was accompanied by a poorer prognosis, regardless of the metastatic spread and with the same treatment.

In the nmOSCC group, a statistically significant (p 0.027) alteration of HOTTIP expression was then evaluated, identified as upregulated also in gastric cancer tissues compared to the levels of the non-tumor counterpart. Furthermore, HOTTIP expression levels were

associated with poor differentiation, advanced TNM stages, and lymph node metastases [129].

Still with reference to this lncRNA, the relationship between HOTTIP expression and overall or disease-free survival in 90 patients with pancreatic carcinoma, after radical resection, was also analyzed. Patients with higher HOTTIP expression had shorter disease-free survival and overall survival than those with lower expression [130].

LSINCT5 (long stress-induced non-coding transcript 5) is dramatically upregulated in breast and ovarian cancer and is closely involved in cell proliferation. The aberrant expression of LSINCT5 was detected in gastrointestinal cancer with respect to adjacent normal tissue samples or with respect to cell lines, using quantitative reverse transcription PCR (RT-qPCR). Numerous studies have also focused on the potential relationship between LSINCT5 tumor levels and the clinical pathological features of gastrointestinal cancer, noting that the increased expression of LSINCT5 was related to a larger tumor size, deeper tumor depth, and an advanced clinical stage [131].

Prostate cancer-associated transcript 1 (PCAT1) has been reported as originally identified as a prostate cancer overexpression lncRNA by RNA sequencing and would contribute to cancer progression through the regulation of a number of target genes.

Alterations in its expression profile are accompanied by the development of a variety of human cancers, including esophageal squamous cell carcinoma (ESCC), lung cancer and hepatocellular carcinoma, acting as a biomarker of poor prognosis. High expression of PCAT1 was observed in ESCC tissues and is associated with poor survival.

Preliminary studies have then found that the overexpression of PCAT1 in ESCC tissues and cell lines was associated with an increase in the proliferation activity of tumor cells, by means of a sponging mechanism against miR-326. Specifically, PCAT1 would function as a ceRNA, sequences with regulatory functions of miRNA activity through base-pairing interactions. By means of a sponge coupling towards miR-326, its action as a tumor suppressor and interaction with KRAS would be determined with

variations in the regulation of AKT and ERK signaling pathways [132].

From the results of our analysis, this lncRNA was strongly overexpressed in the OSCC samples, as also found in the literature with regard to tissues associated with esophageal squamous cell carcinomas (ESCC). In this sense, given the proximity of the causal factors, further molecular biology studies to evaluate a possible common pathway of carcinogenic development associated with the same alterations.

Growing evidence has suggested that non-coding prostate cancer RNA 1 (PRNCR1) may participate in the pathogenesis of non-small cell lung cancer (NSCLC). In fact, numerous studies have found PRNCR1 as upregulated in tissues and cell lines of NSCLC. In this case a specific interaction was also found with a molecule not encoded by miR-126-5p, being a tumor suppressor in multiple tumors of multiple anatomical districts and regulator the mechanisms of proliferation and invasion of tumor cells [133].

SPRY4-IT1 (intron transcript SPRY4 1), is a lncRNA derived from an intron within the SPRY4 gene, identified

as responsible for the development of multiple tumors. Specifically, in various studies, an alteration of the expression profile in gastric cancer samples was measured in a negative sense compared to the control tissues, allowing this molecule to associate with tumor suppressor activity. The reduced expression of this lncRNA, in fact, was associated with larger tumor size, advanced pathological stage, greater depth of invasion and lymphatic metastases and in general, patients with lower expression of SPRY4-IT1 were associated with a relatively poor prognosis. DNA methylation may be a key factor in controlling SPRY4-IT1 expression. Furthermore, SPRY4-IT1 would contribute to the development of gastric cancer cell metastases in part through the regulation of the epithelial-mesenchymal transition process (EMT) [134].

The presence of a SPRY4-IT1 overexpression, within the nmOSCC sample examined in our study, could be associated with the presence of regulatory mechanisms inhibiting cancer development, motivating the absence of a neoplastic behavioral profile aimed at metastasis

and correlating with the small lesion size and low staging.

Ultimately, the overall expression profile highlighted as significant in the OVC group was on the whole well differentiated from the profile that emerged in the other two groups (mOSCC and nmOSCC). In the event that a constant alteration of the expression profile was ascertained in this histological subtype of oral cancer, its early definition in the biopsy phase of early neoplastic stages could have important implications on both the type of treatment and therapeutic approach in general.

AIRN lncRNA has been investigated in 2 studies in the literature for its possible protective and restorative role against podocytes in relation to lesions induced by diabetic nephropathy by binding to Igf2bp2. While certainly falling into different areas and not associated with carcinogenesis, the overexpression of this lncRNA still facilitates cellular repair pathways and the vitality of podocytes, protecting them from apoptosis [135].

In our study, AIRN overexpression was found in the OVC group (p 0.009), therefore not statistically

associated with metastases and a generally better prognosis.

The overexpression of GACAT1 has been evaluated in numerous studies referring to various anatomical districts, and associated with the promotion, proliferation, invasion and migration of neoplastic cells, specifically with regard to gastric carcinoma (GC). It was in fact confirmed that the lncRNA GACAT1 was upregulated, in a comparison study among 57 tissue samples GC tissues and cellular samples, also revealing that the knockdown of GACAT1 significantly suppressed the proliferation, invasion and migration of cells GC. The results of this study suggested that GACAT1 may act as an oncogene in GC and its overexpression may contribute to tumorigenesis and neoplastic progression [136].

The results that can be deduced from the quantification of this lncRNA in the OVC group, by investigation with RT PCR, express, in this group, hypoexpression values compared to the control tissue. This reduced expression could explain the behavior of this type of neoplasm, characterized by local aggression but at the same time

characterized by a low tendency to metastasize and to be able to relate and differentiate it from the expression profile and behavior of the OSCC.

Long non-coding RNA (lncRNA) HIF1A-AS1 has been identified as capable of playing important regulatory roles in cancer biology, and its upregulation has been measured in several cancers such as glioblastoma, breast cancer, in a number of renal tumors, as well as in non-small cell lung cancer (NSCLC), through the interaction between the HIF1A-AS1 complex and apoptotic proteins, playing a key role in proliferation and apoptosis [137].

With reference to our study, the limit linked to the use of "apparently" healthy control samples at a standardized distance of 1.5 mm - which could be affected by field carcinization phenomena - certainly deserves to be mentioned.

Secondly, it would be necessary to both introduce and use more specific test kits for squamous cell carcinomas of the head and neck area, which can effectively evaluate any pathways of alteration of the common

expression profile among the various neoplasms. On the basis of the data obtained, and in function of the differential expression in the various subgroups analyzed, it has been possible to infer the presence of characterizing profiles.

Any future studies could further enrich and refine the mapping and the understanding of the altered expression profile of these feared neoplasms.The possible analysis of the expression profile of these lncRNAs in early diagnostic phases of an incisional biopsy type, in comparison with the expression profile of the tissues of a possible surgical piece, could provide clinical and therapeutic indications that could be standardized and may result being extremely useful.

6. CONCLUSIONS

Although treatment options for OSCC patients have improved in recent decades, the overall survival rate is still low, underscoring the importance of expanding research into new treatment options. We have turned our attention to the differential study of the expression profile of some possible tumor biomarkers between different subtypes of oral malignant neoplasms, associated with different behaviours, compared to healthy control tissues, to allow possible correlations on a diagnostic and possibly clinical level. The processing of the data emerging from the molecular biology analysis with RT PCR, in multivariate analysis with two-component variance maximization, demonstrated a wide variety of profiles between the samples under examination (mOSCC, nmOSCC, OVC) and - more generally - each group is characterized by the dysregulation of several lncRNAs.

In the group of OSCC associated with metastases, the lncRNAs LINC00312, PRNCR1, XIST X, could

represent, given their characteristic oncogenic action found in numerous other neoplasms, possible early indicators of an evolution in a metastatic sense, with possible implications in terms of staging.

As regards the group of OSCCs not associated with metastases, lncRNA HOTAIR, PCAT1, SPRY4-IT1 were found as possible biomarkers.

The two HOTAIR lncRNAs, PCAT1 have emerged as important oncogenes remarkably expressed in several squamous tumoral cells of the head and neck district (HNSCC). They could also be configured as prognostic indicators at the oral level, even in cases not necessarily associated with metastatic spread, as demonstrated in our study.

SPRY4-IT1 lncRNA, overexpressed in the group of oral cancer not associated with metastatic spread, has been studied in the gastroenterological field for its onco-suppressive action against gastric cancer. Reduced expression of this lncRNA, in fact, has been associated with larger tumoral size, advanced disease stage, greater depth of invasion and lymphatic metastases.

Ultimately, in the OVC group, a reduced expression of GACAT1 was measured, which could significantly explain the reduced proliferation, invasion, and cell migration, all of them being characteristics of this histotype.

All these results, in their totality, lead to a new world view of contemporary oncology, including the need to integrate the knowledge acquired in Proteomics, Genomics, Transcriptomics and Metabolomics to epigenetic events, for which the main characters are long transcripts non-coding RNA (lncRNA). The interaction of different ncRNAs and lncRNAs in the different phases of cell biology could help in the development of new therapeutic and clinical aids if the regulatory dynamics of these transcripts were better defined.

Further studies are therefore necessary for a more precise definition of the expression pattern of these neoplasms for a greater understanding of this labyrinth made of genes and molecules once defined as junk.

7. BIBLIOGRAPHY

1. Global Burden of Disease Cancer Collaboration. *Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability- adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the global burden of disease study*. JAMA Oncol. 2017;3(4):524–548
2. Vigneswaran N, Williams MD. 2014. *Epidemiologic trends in head and neck cancer and aids in diagnosis.*OralMaxillofacSurgClin North Am. 26(2):123–141.
3. Chi AC, Day TA, Neville BW. *Oral cavity and oropharyngeal squamous cell carcinoma--an update*. CA Cancer J Clin. 2015;65(5):401–21.
4. Pannone G, Santoro A, Papagerakis S, Lo Muzio L, De Rosa G, Bufo P. *The role of human papillomavirus in the pathogenesis of head & neck squamous cell carcinoma: an overview.* Infect Agent Cancer. 2011;6(1):4.
5. Sankunny M, Parikh RA, Lewis DW, Gooding WE, Saunders WS, Gollin SM. *Targeted inhibition of ATR or CHEK1 reverses radioresistance in oral squamous cell carcinoma cells with distal chromosome arm 11q loss*. GenesChromosomesCancer. 2014;53(2):129–43.
6. Mehrotra R, Yadav S. *Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations.* Indian J Cancer. 2006;43(2):60–6.
7. Patel SC, Carpenter WR, Tyree S, Couch ME, Weissler M, Hackman T, Hayes DN, Shores C, Chera BS. *Increasing incidence of oral tongue squamous cell carcinoma in young white women, age 18 to 44 years*. J ClinOncol. 2011;29(11):1488–94.
8. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, Soerjomataram I. 2017. *The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012*. CA Cancer J Clin. 67(1):51–64.
9. MooreSR,JohnsonNW,PierceAM,WilsonDF:*Theepidemiology of mouth cancer: a review of global incidence*. Oral Dis.2000 Mar; 6(2):65-74.
10. Rodriguez T et al. *Risk factors for oral and pharyngeal cancer in young adults*. OralOncol. 2004; 40(2):207-13.
11. Abu-Ghanem S, Yehuda M, Carmel NN, Leshno M, Abergel A, Gutfeld O, Fliss DM. *Elective Neck Dissection vs Observation in Early-Stage Squamous Cell Carcinoma of the Oral Tongue With No Clinically Apparent*

Lymph Node Metastasis in the Neck: A Systematic Review and Meta-analysis. JAMA Otolaryngol Head NeckSurg. 2016; 142:857–865.

12. Hema KN, Smitha T, Sheethal HS, Mirnalini SA. 2017. *Epigenetics in oral squamous cell carcinoma*. J Oral MaxillofacPathol. 21(2):252–259.

13. Le Campion ACOV, Ribeiro CMB, Luiz RR, da Silva Júnior FF, Barros HCS, Dos Santos KCB, Ferreira SJ, Gonçalves LS, Ferreira SMS. 2017. *Low survival rates of oral and oropharyngeal squamous cell carcinoma*. Int J Dent. 2017:5815493.

14. Bitu CC, Destro MF, Carrera M, da Silva SD, Graner E, Kowalski LP, Soares FA, Coletta RD. 2012. Hoxa1 is overexpressed in oral squamous cell carcinomas and its expression is correlated with poor prognosis. BMC Cancer. 12:146.

15. A. J. Atkinson, W. A. Colburn, V. G. deGruttola et al., "*Biomarkers and surrogate endpoints: preferred definitions and conceptual framework," Clinical Pharmacology & Therapeutics, vol. 69, no. 3, pp. 89–95, 2001*

16. Guidance for Industry-E15 Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding
Categories, http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129296.pdf.

17. D. C. Anderson and K. Kodukula: *Biomarkers in pharmacology and drug discovery*, Biochemical Pharmacology, vol. 87, no. 1, pp. 172–188, 2014

18. R. Frank and R. Hargreaves: *Clinical biomarkers in drug discovery and developmen*t, Nature Reviews Drug Discovery, vol. 2, no. 7, pp. 566–580, 2003.

19. S. Anfossi, A. Babayan, K. Pantel and a. George: *Calin Clinical utility of circulating non- coding RNAs, an update;* Int J Oncol 2009;10:743-23

20. Ramos-Garcia P, Gil-Montoya JA, Scully C, Ayen A, Gonzalez-Ruiz L, Navarro- Trivino FJ, Gonzalez-Moles MA. 2017. *An update on the implications of cyclin D1 in oral carcinogenesis.*OralDis. 23(7):897–912.

21. Lingen MW, Pinto A, Mendes RA, Franchini R, Czerninski R, Tilakaratne WM, Partridge M, Peterson DE, Woo SB. 2011. *Genetics/epigenetics of oral premalignancy: current status and future research*. OralDis. 17(Suppl 1):7–22.

22. Wong N, Khwaja SS, Baker CM, Gay HA, Thorstad WL, Daly MD, Lewis JS Jr, Wang X; *Prognostic microRNA signatures derived from the cancer genome atlas for head and neck squamous cell carcinomas.* 2016 Cancer Med. 5(7):1619–1628.

23. C. M. Pereira et al; *miRNAs: Important Targets for Oral Cancer Pain Researc*h; Hindawi BioMed Research International Volume 2017, Article ID 4043516

24. Siegel, R. L., Miller, K. D., and Jemal, A. (2019). *Cancer statistics, 2019*. *CA Cancer J. Clin.* 69, 7–34. doi: 10.3322/caac.21551

25. Scully C, Moles DR. *Oral cancer*. In: Heggenhougen K, Quah S, editors. International Encyclopedia of Public Health, vol. 4. San Diego: Academic Press; 2008. p. 668–77.

26. Petti S. *Lifestyle risk factors for oral cancer*. Oral Oncol 2009;45(4–5):340–50.

27. Campisi G, Panzarella V, Giuliani M, Lajolo C, Di Fede O, Falaschini S, Di Liberto C, Scully C, Lo Muzio L. *Human papillomavirus: its identity and controversial role in oral oncogenesis, premalignant and malignant lesions (review*). Int J Oncol2007;30:813-23

28. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. *Case-control study of human papillomavirus and oropharyngeal cancer*. N Engl J Med 2007;356(19):1944–56.

29. Bouda M, Gorgoulis VC, Kastrinakis NG, Giannoudis A, Tsoli E, Danassi-Afentaki D, Foukas P, Kyroudi A, Laskaris G, Herrington CS, Kittas C. "*High risk" HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal oral mucosa*. Mod Pathol2000;13:644-53

30. Thomson PJ, Hamadah O*. Cancerisation within the oral cavity: the use of 'field mapping biopsies' in clinical management*. Oral Oncol 2007;43(1):20–6.

31. Meyer MS, Joshipura K, Giovannucci E, Michaud DS. *A review of the relationship between tooth loss, periodontal disease, and cancer.*CancerCauses Control 2008;19(9):895–907.

32. Bloching M, Reich W, Schubert J, Grummt T, Sandner A. *The influence of oral hygiene on salivary quality in the Ames test, as a marker for genotoxic effects*. Oral Oncol 2007;43(9):933–9.

33. Derk CT, Rasheed M, Spiegel JR, Jimenez SA. *Increased incidence of carcinoma of the tongue in patients with systemic sclerosis*. J Rheumatol 2005;32(4):637–41.

34. Fialka F, Gruber RM, Hitt R, Opitz L, Brunner E, Schliephake H, Kramer FJ: *CPA6, FMO2, LGI1, SIAT1 and TNC are differentially expressed in early- and late-stage oral squamous cell carcinoma a pilot study*. Oral Oncol 2008, 44(10):941-948.

35. Chiang WF, Liu SY, Yen CY, Lin CN, Chen YC, Lin SC, Chang KW: *Association of epidermal growth factor receptor (EGFR) gene copy number amplification with neck lymph node metastasis in areca-associated oral carcinomas*. Oral Oncol 2008, 44(3):270-276.

36. Serefoglou Z, Yapijakis C, Nkenke E, Vairaktaris E: *Genetic association of cytokine DNA polymorphisms with head and neck cancer*. Oral Oncol 2008, 44(12):1093-1099.

37. Gupta R, Sharma SC, Das SN: *Association of TNF-alpha and TNFR1 promoters and 30 UTR region of TNFR2 gene polymorphisms with genetic susceptibility to tobacco-related oral carcinoma in Asian Indians.* Oral Oncol 2008, 44(5):455-463.

38. Coutinho Camillo CM, Lourenco SV, Nishimoto IN, Kowalski LP, Soares FA: *Expression of Bcl-2 family proteins and association with clinicopathological characteristics of oral squamous cell carcinoma.* Histopathology 2010, 57:304-316.

39. Chaudhary AK, Singh M, Bharti AC, Singh M, Shukla S, Singh AK, Mehrotra R: *Synergistic effect of stromelysin-1(matrix metalloproteinase-3)promoter (-117 5 A- > 6A) polymorphism in oral submucous fibrosis and head and neck lesions*. BMC CANCER 2010, 10:369.

40. Gillison ML. *Current topics in the epidemiology of oral cavity and oropharyngeal cancers*. Head Neck 2007;29:779-92

41. Warnakulasuriya S, Sutherland G, Scully C. *Tobacco, oral cancer and treatment of dependence*. Oral Oncol 2005;41:244-60

42. Llewellyn CD, Johnson NW, Warnakulasuriya KA. *Risk factors for oral cancer in newly diagnosed patients aged 45 years and younger: a case-control study in Southern England.*J OralPatholMed2004;33:525-32

43. Wynder EL, Mushinski MH, Spivak JC. *Tobacco and alcohol consumption in relation to the development of multiple primary cancers*. Cancer 1977;40:1872-78

44. Groome PA, Schulze K, Boysen M, Hall SF, Mackillop WJ. *A comparison of published head and neck stage groupings in carcinomas of the oral cavity*. Head Neck 2001;23:613-24

45. Woolgar JA. *Histological distribution of cervical lymph node metastases from intraoral/oropharyngeal squamous cell carcinomas*. Br J OralMaxillofacSurg1999;37:175-80

46. Barthélémy I, Sannajust JP, Revol P, Mondié JM. *Oral cancer. Preamble, epidemiology, clinical study*. EMC-Stomatologie2005;1:277-94

47. Robbins KT, Clayman G, Levine PA, Medina J, Sessions R, Shaha A, Som P, Wolf GT. *Neck dissection classification update: revisions proposed by the American Head and Neck Society and the American*

Academy of Otolaryngology-Head and Neck Surgery. Arch Otolaryngol Head Neck Surg 2002;128:751-8

48. Shah JP. *Cancer of the Head and Neck*. London: BC Decker Inc Hamilton; 2001
49. Ward GE, Robben JO. *A composite operation for radical neck dissection and removal of cancer of the mouth.*Cancer 1951;4:98-109
50. Reuther T, Schuster T, Mende U, Kubler A. *Osteoradionecrosis of the jaws as a side effect of radiotherapy of head and neck tumour patients. A report of a thirty year retrospective study.* Int J Oral Maxillofac Surg 2003;32:289-95
51. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, Kish JA, Kim HE, Cmelak AJ, Rotman M, et al. *Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck.* N Engl J Med 2004;350:1937-44
52. Ackerman LV: Verrucous carcinoma of the oral cavity. Surgery 23: 670-678, 1948.
53. Chaisuparat R, Limpiwatana S, Kongpanitkul S, Yodsanga S and Jham BC: The Akt/mTOR pathway is activated inverrucous carcinoma of the oral cavity. J Oral Pathol Med: Jan 17, 2016
54. Kamala K, Sankethguddad S and Sujith SG: Verrucous carcinoma of oral cavity - a case report with review of literature. IJhSR 5:330-334, 2015.
55. Gokavarapu S, Parvataneni N, Charan CR, Puthamakula S, Kulkarni G and Reddy BS: Multi centricity of oral verrucouscarcinoma: A case series of 22 cases. Indian J Otolaryngol head Neck Surg 67: 138-142, 2015.
56. Pravda C, Srinivasan h, Koteeswaran D and Manohar LA: Verrucous carcinoma in association with oral submucousfibrosis. Indian J Dent Res 22: 615, 2011.
57. Agnihotri A and Agnihotri D: Verrucous carcinoma: A study of 10 cases. Indian J Oral Sci 3: 79-83, 2012.
58. Gupta S, Kumar K, Raviprakash SM and Arunkumar KV: Ackerman's tumor of the oral cavity: A study of four cases withits conglomerate appearance. J Dent Specialities 3: 92-95, 2015.
59. Noble-Topham SE, Fliss DM, hartwick RW, McLachlin CM, Freeman JL, Noyek AM and Andrulis IL: Detection and typing of human papillomavirus in verrucous carcinoma of the oral cavity using the polymerase chain reaction. ArchOtolaryngol head Neck Surg 119: 1299-1304, 1993.
60. de Spíndula-Filho JV, da Cruz AD, Oton-Leite AF, Batista AC, Leles CR, de CássiaGonçalvesAlencar R, Saddi VA and Mendonça EF: Oral squamous cell carcinoma versus oral verrucous carcinoma: An approach

to cellular proliferation andnegative relation to human papillomavirus (hPV). Tumour Biol 32: 409-416, 2011.

61. Waskowska J, Koszowski R, Raczkowska-Siostrzonek A and Stemplewska K: Verrucous carcinoma of the tongue-a rarecase study. Cent Eur J Med 7: 145-148, 2012.

62. Rodrigues J, Vaz OP, Salelkar RS, Ramani A, Falari S and VeereshhM: A rare case of verrucous carcinoma on the dorsumof the tongue. Int J Adv Case Rep 2: 530-531, 2015.

63. Alkan A, Bulut E, Gunhan O and Ozden B: Oral verrucous carcinoma: A study of 12 cases. Eur J Dent 4: 202-207, 2010.

64. Liu O, zhang h, Wang Y, Quan h, zhang J, zhou J, zuo J, Tang J, Fang X, Wang W, *et al*: Stereology study of oralverrucous carcinoma. J BUON 17: 343-349, 2012.

65. KaragozogluKh, Buter J, Leemans CR and Rietveld Dh, van den Vijfeijken S and van der Waal I: Subset of patients with verrucous carcinoma of the oral cavity benefit from treatment with methotrexate. Br J Oral Maxillofac Surg 50: 513-518,2012.

66. Tang zG, Li JY, Su T, Yao zG, Shen zh and Li hB: The clinic study on oral verrucous carcinoma. J Oral Maxillofac Surg12: 87-88, 2002.

67. Rahali L, Omor Y, Mouden K, Mahdi Y, Elkacemi h, Elmajjaoui S, Latib R, Kebdani T, Boujida MN and Benjaafar N: Oral verrucous carcinoma complicating a repetitive injury by the dental prosthesis: A case report. Pan Afr Med J 20: 297, 2015.

68. Evans JR, Feng FY, Chinnaiyan AM. *The bright side of dark matter: lncRNAs in cancer.*J ClinInvest. 2016; 126:2775–2782

69. Mercer TR, Dinger ME, Mattick JS. *Long non-coding RNAs: insights into functions.*NatRevGenet. 2009;10(3): 155–159.

70. Novikova IV, Hennelly SP, Sanbonmatsu KY. *Sizing up long non-coding RNAs: do lncRNAs have secondary and tertiary structure?*Bioarchitecture. 2012;2(6):189–99.

71. Ingolia NT, Lareau LF, Weissman JS. *Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes*. Cell. 2011;147(4): 789 – 802.

72. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL: *Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses*. Genes Dev 2011, 25:1915–1927.

73. Batista PJ, Chang HY: *Long noncoding RNAs: cellular address codes in development and disease*. Cell 2013, 152:1298–1307.

74. Ma L, Bajic VB, Zhang Z. *On the classification of long non-coding RNAs.* RNA Biol. 2013; 10 (6): 924–33

75. Bertani S, Sauer S, Bolotin E, Sauer F*: The noncoding RNA Mistral activates Hoxa6 and Hoxa7 expression and stem cell differentiation by recruiting MLL1 to chromatin*. Mol Cell 2011, 43:1040–1046.

76. Li W, Notani D, Ma Q, Tanasa B, Nunez E, Chen AY, Merkurjev D, Zhang J, Ohgi K, Song X, Oh S, Kim HS, Glass CK, Rosenfeld MG: *Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation.* Nature 2013, 498:516–520.

77. Barry G, Briggs JA, Vanichkina DP, Poth EM, Beveridge NJ, Ratnu VS, Nayler SP, Nones K, Hu J, Bredy TW, Nakagawa S, Rigo F, Taft RJ, Cairns MJ, Blackshaw S, Wolvetang EJ, Mattick JS: *The long non-coding RNA Gomafu is acutely regulated in response to neuronal activation and involved in schizophrenia-associated alternative splicing.* Mol Psychiatry 2013, 19:486–494.

78. Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, Lajoie BR, Protacio A, Flynn RA, Gupta RA, Wysocka J, Lei M, Dekker J, Helms JA, Chang HY: *A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression*. Nature 2011, 472:120–124.

79. Mao YS, Sunwoo H, Zhang B, Spector DL: *Direct visualization of the co-transcriptional assembly of a nuclear body by noncoding RNAs*. Nat Cell Biol 2011, 13:95–101.

80. Yang L, Lin C, Liu W, Zhang J, Ohgi KA, Grinstein JD, Dorrestein PC, Rosenfeld MG: *ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs*. Cell 2011, 147:773–788.

81. Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, Huarte M, Zhan M, Becker KG, Gorospe M: *LincRNA-p21 suppresses target mRNA translation*. Mol Cell 2012, 47:648–655.

82. Kretz M, Siprashvili Z, Chu C, Webster DE, Zehnder A, Qu K, Lee CS, Flockhart RJ, Groff AF, Chow J, Johnston D, Kim GE, Spitale RC, Flynn RA, Zheng GX, Aiyer S, Raj A, Rinn JL, Chang HY, Khavari PA: *Control of somatic tissue differentiation by the long non-coding RNA TINCR*.Nature 2013, 493:231–235.

83. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N: *Circular RNAs are a*

large class of animal RNAs with regulatory potency. Nature 2013, 495:333–338.

84. Sauvageau M, Goff LA, Lodato S, Bonev B, Groff AF, Gerhardinger C, Sanchez-Gomez DB, Hacisuleyman E, Li E, Spence M, Liapis SC, Mallard W, Morse M, Swerdel MR, D'Ecclessis MF, Moore JC, Lai V, Gong G, Yancopoulos GD, Frendewey D, Kellis M, Hart RP, Valenzuela DM, Arlotta P, Rinn JL: *Multiple knockout mouse models reveal lincRNAs are required for life and brain development*. eLife 2013, 2:e01749

85. Wu Y, Zhang L, Zhang L, Wang Y, Li H, Ren X, Wei F, Yu W, Liu T, Wang X, Zhou X, Yu J, Hao X: *Long non- coding rnahotair promotes tumor cell invasion and metastasis by recruiting ezh2 and repressing e-cadherin in oral squamous cell carcinoma.* Int J Oncol 2015;46:2586-2594.

86. Zou, A. E., Ku, J., Honda, T. K., Yu, V., Kuo, S. Z., Zheng, H., ... Ongkeko, W. M. (2015). *Transcriptome sequencing uncovers novel long noncoding and small nucleolar RNAs dysregulated in head and neck squamous cell carcinoma*. *RNA (New York, N.Y.)*, *21*, 1122–1134. https://doi. org/10.1261/rna.049262.114

87. Tang, H., Wu, Z., Zhang, J., & Su, B. (2013). *Salivary lncRNA as a potential marker for oral squamous cell carcinoma diagnosis.Molecular Medicine Reports*, *7*, 761–766. https://doi.org/10.3892/mmr.2012.1254

88. Zhou X, Chen J, Tang W. *The molecular mechanism of HOTAIR in tumorigenesis, metastasis, and drug resistance.* Acta BiochimBiophys Sin (Shanghai). 2014; 46:1011– 1015.

89. Loewen G, Jayawickramarajah J, Zhuo Y, Shan B. *Functions of lncRNA HOTAIR in lung cancer.* J Hematol Oncol. 2014; 7:90.

90. Arunkumar, G., Deva MagendhraRao, A. K., Manikandan, M., Arun, K., Vinothkumar, V., Revathidevi, S., ... Munirajan, A. K. (2017). *Expression profiling of long non-coding RNA identifies linc-RoR as a prognostic biomarker in oral cancer*. *TumorBiology*, *39*, 1–11. 101042831769836

91. Lu, M. Y., Liao, Y. W., Chen, P. Y., Hsieh, P. L., Fang, C. Y., Wu, C. Y. Tsai, L. L. (2017). *Targeting LncRNA HOTAIR suppresses cancer stemness and metastasis in oral carcinomas stem cells through modulation of EMT*. *Oncotarget*, *8*, 98542–98552.

92. Gibb, E. A., Enfield, K. S., Stewart, G. L., Lonergan, K. M., Chari, R., Ng, R. T., ... Lam, W. L. (2011). *Long non-coding RNAs are ex- pressed in oral mucosa and altered in oral premalignant lesions*. Oral Oncology, 47, 1055–1061. https://doi.org/10.1016/j. oraloncology.2011.07.008

93. Liang, J., Liang, L., Ouyang, K., Li, Z., & Yi, X. (2017). *MALAT1 induces tongue cancer cells' EMT and inhibits apoptosis through Wnt/beta- catenin*

signaling pathway. Journal of Oral Pathology and Medicine, 46, 98–105. https://doi.org/10.1111/jop.12466

94. Huang, G., He, X., & Wei, X. L. (2018). *lncRNA NEAT1 promotes cell proliferation and invasion by regulating miR365/RGS20 in oral squamous cell carcinoma.Oncology Reports*, *39*, 1948–1956.

95. Liu, Z., Wu, C., Xie, N., & Wang, P. (2017). *Long non-coding RNA MEG3 inhibits the proliferation and metastasis of oral squamous cell carcinoma by regulating the WNT/beta-catenin signaling path- way.*Oncology Letters, *14*, 4053–4058. https://doi.org/10.3892/ ol.2017.6682

96. Fang, Z., Zhao, J., Xie, W., Sun, Q., Wang, H., &Qiao, B. (2017). *LncRNA UCA1 promotes proliferation and cisplatin resistance of oral squamous cell carcinoma by sunppressing miR-184 expression.*Cancer Medicine, *6*, 2897–2908. https://doi.org/10.1002/ cam4.1253

97. Peng, C. H., Liao, C. T., Peng, S. C., Chen, Y. J., Cheng, A. J., Juang, J. L.,Yen, T. C. (2011). *A novel molecular signature identified by sys- tems genetics approach predicts prognosis in oral squamous cell carcinoma*. *PLoS ONE*, *6*, e23452. https://doi.org/10.1371/journal. pone.0023452

98. Ma, Y., Hu, X., Shang, C., Zhong, M., & Guo, Y. (2017). *Silencing of long non-coding RNA CCAT2 depressed malignancy of oral squamous cell carcinoma via Wnt/beta-catenin pathway*. Tumour Biology, *39*, 1–9. https://doi.org/10.1177/1010428317717670

99. Gao, L., Ren, W., Zhang, L., Li, S., Kong, X., Zhang, H., ... Zhi, K. (2017). *PTENp1, a natural sponge of miR-21, mediates PTEN ex- pression to inhibit the proliferation of oral squamous cell carcinoma.*Molecular Carcinogenesis, *56*, 1322–1334. https://doi. org/10.1002/mc.22594

100. Brunner, A. L., Beck, A. H., Edris, B., Sweeney, R. T., Zhu, S. X., Li, R., West, R. B. (2012). *Transcriptional profiling of long non-coding RNAs and novel transcribed regions across a diverse panel of archived human cancers*. GenomeBiology, *13*, R75. https://doi.org/10.1186/ gb-2012-13-8-r75

101. Arunkumar, G., Murugan, A. K., Prasanna Srinivasa Rao, H., Subbiah, S., Rajaraman, R., &Munirajan, A. K. (2017). *Long non-coding RNA CCAT1 is overexpressed in oral squamous cell carcinomas and predicts poor prognosis*. Biomedical Reports, *6*, 455–462. https://doi. org/10.3892/br.2017.876

102. Tang, H., Wu, Z., Zhang, J., & Su, B. (2013). *Salivary lncRNA as a potential marker for oral squamous cell carcinoma diagnosis.*Molecular Medicine Reports, *7*, 761–766. https://doi.org/10.3892/mmr.2012.1254

103. Fang, Z., Wu, L., Wang, L., Yang, Y., Meng, Y., & Yang, H. (2014). *Increased expression of the long non-coding RNA UCA1 in tongue squamous cell carcinomas: A possible correlation with cancer metastasis.*Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology, 117, 89–95. https://doi.org/10.1016/j.oooo.2013.09.007

104. Sun, T., Tang, L., & Zhang, M. (2018). *Long noncoding RNA LncEGFR promotes cell proliferation and inhibits cell apoptosis via regulating the expression of EGFR in human tongue cancer.*Molecular Medicine Reports, *17*, 1847–1854.

105. Guo, Y., Ma, Y., Hu, X., Song, R., Zhu, L., & Zhong, M. (2018). *Long non-coding RNA CEBPA-AS1 correlates with poor prognosis and pro- motes tumorigenesis via CEBPA/Bcl2 in oral squamous cell carcinoma.*Cancer Biology & Therapy, *19*, 205–213. https://doi.org/10.10 80/15384047.2017.1416276

106. Zhu, G., Wang, S., Chen, J., Wang, Z., Liang, X., Wang, X.,Li, L. (2017). *Long noncoding RNA HAS2-AS1 mediates hypoxia-induced invasive-ness of oral squamous cell carcinoma.*MolecularCarcinogenesis, *56*, 2210–2222. https://doi.org/10.1002/mc.22674

107. Yu, J., Liu, Y., Guo, C., Zhang, S., Gong, Z., Tang, Y., ... Yang, X. (2017). *Upregulated long non-coding RNA LINC00152 expression is associated with progression and poor prognosis of tongue squamous cell carcinoma.* Journal of Cancer, *8*, 523–530. https://doi.org/10.7150/ jca.17510

108. Wang, Y., Zhang, X., Wang, Z., Hu, Q., Wu, J., Li, Y., ... Cheng, B. (2018). *LncRNA-p23154 promotes the invasion-metastasis potential of oral squamous cell carcinoma by regulating Glut1-mediated glycolysis*. Cancer Letters, *434*, 172–183. https://doi.org/10.1016/j. canlet.2018.07.016

109. Huang, W., Cui, X., Chen, J., Feng, Y., Song, E., Li, J., &Liu, Y. (2016). *Long non-coding RNA NKILA inhibits migration and invasion of tongue squamous cell carcinoma cells via suppressing epithelial- mesenchymal transition.*Oncotarget, *7*, 62520–62532.

110. Hong, Y., He, H., Sui, W., Zhang, J., Zhang, S., & Yang, D. (2018). *Long non-coding RNA H1 promotes cell proliferation and invasion by acting as a ceRNA of miR138 and releasing EZH2 in oral squamous cell carcinoma.*International Journal of Oncology, *52*, 901–912.

111. Wu, J., &Xie, H. (2015). *Expression of long noncoding RNA-HOX tran-script antisense intergenic RNA in oral squamous cell carcinoma and effect on cellgrowth*. *TumourBiology*, *36*, 8573–8578. https://doi. org/10.1007/s13277-015-3598-5

112. Wright, M. W., and Bruford, E. A. J. H. (2011). *Naming'junk': human non-protein coding RNA (ncRNA) gene nomenclature.*Hum. Genomics5:90. doi: 10.1186/ 1479- 7364- 5- 2- 90

113. Rinn, J. L., and Chang, H. Y. (2012). *Genome regulation by long noncoding RNAs*. Annu. Rev. Biochem. 81, 145–166. doi: 10.1146/annurev- biochem- 051410- 092902

114. Li, S., Li, B., Zheng, Y., Li, M., Shi, L., and Pu, X. (2017). *Exploring functions of long noncoding RNAs across multiple cancers through co-expression network*. Sci. Rep.7:754.

115. Salviano-Silva, A., Lobo-Alves, S. C., Almeida, R. C., Malheiros, D., and Petzl- Erler, M. L. (2018). *Besides pathology: long non-coding RNA in cell and tissue homeostasis.*Noncoding RNA4. doi: 10.3390/ncrna4010003

116. Bao, S., Zhao, H., Yuan, J., Fan, D., Zhang, Z., Su, J., et al. (2019). *Computational identification of mutator-derived lncRNA signatures of genome instability for improving the clinical outcome of cancers: a case study in breast cancer.*Brief Bioinform. doi: 10.1093/bib/bbz118

117. Perez-Sayans, M., Somoza-Martin, J. M., Barros-Angueira, F., Reboiras-Lopez, M. D., Gandara Rey, J. M., and Garcia-Garcia, A. (2009). *Genetic and molecular alterations associated with oral squamous cell cancer (Review).*Oncol. Rep. 22, 1277–1282. doi: 10.3892/or_00000565

118. Sun, J., Zhang, Z., Bao, S., Yan, C., Hou, P., Wu, N., et al. (2020). Identification of tumor immune infiltration-associated lncRNAs for improving prognosis and immunotherapy response of patients with non-small cell lung cancer. *J. Immunother. Cancer* 8:e000110. doi: 10.1136/jitc-2019-000110

119. Peng, Zhenzi et al. *The Long Noncoding RNA LINC00312 Induces Lung Adenocarcinoma Migration and Vasculogenic Mimicry through Directly Binding YBX1.*Molecular cancer 17.1 (2018): 167–6. Web.

120. Zhang, Wenling et al*. Expression of LINC00312, a Long Intergenic Non-Coding RNA, Is Negatively Correlated with Tumor Size but Positively Correlated with Lymph Node Metastasis in Nasopharyngeal Carcinoma.*Journal of Molecular Histology 44.5 (2013): 545–554. Web.

121. Zheng, Haitao et al. *BRAF-Activated Long Noncoding RNA Modulates Papillary Thyroid Carcinoma Cell Proliferation through Regulating Thyroid Stimulating Hormone Recepto*r. Cancer research and treatment 48.2 (2016): 698–707. Web.

122. Abdollahzadeh, Rasoul et al. *Expression and Clinicopathological Significance of AOC4P, PRNCR1, and PCAT1 lncRNAs in Breast Cancer.*Pathology, research and practice 216.10 (2020): 153131–. Web.

123. Yue, Lei, and Jing Guo. *LncRNA TUSC7 Suppresses Pancreatic Carcinoma Progression by Modulating miR-371a-5p Expression.* Journal of cellular physiology 234.9 (2019): 15911–15921. Web.

124. Zhang, Xiu-Tian et al. *Long Non-Coding RNA (lncRNA) X-Inactive Specific Transcript (XIST) Plays a Critical Role in Predicting Clinical Prognosis and Progression of Colorectal Cancer.*Medical science monitor 25 (2019): 6429–6435. Web.

125. Li, Zhixin et al. *GNAS-AS1/miR-4319/NECAB3 Axis Promotes Migration and Invasion of Non-Small Cell Lung Cancer Cells by Altering Macrophage Polarization.*Functional & integrative genomics 20.1 (2019): 17–28. Web.

126. Li J, Wang J, Zhong Y, et al. *HOTAIR: a key regulator in gynecologic cancers.* Cancer Cell Int. 2017;**17**:65.

127. Gupta RA, Shah N, Wang KC, et al. *Long noncoding RNA HOTAIR reprograms chromatin state to promote cancer metastasis*. Nature. 2010;464:1071-1076.

128. Yang Z, Zhou L, Wu LM, et al. *Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation.* AnnSurgOncol. 2011;18(5):1243-1250.

129. Zhao, Rui et al. Exosomal*Long Noncoding RNA HOTTIP as Potential Novel Diagnostic and Prognostic Biomarker Test for Gastric Cancer. Molecular cancer* 17.1 (2018): 68–5.

130. Fu, Zhiqiang et al. *LncRNA HOTTIP Modulates Cancer Stem Cell Properties in Human Pancreatic Cancer by Regulating HOXA9.* Cancer letters 410 (2017): 68–81.

131. Xu, Mi-Die et al. *Long Non-Coding RNA LSINCT5 Predicts Negative Prognosis and Exhibits Oncogenic Activity in Gastric Cancer.*Medicine (Baltimore) 93.28 (2014): e303–. Web.

132. Huang, Lijie et al. *Long Noncoding RNA PCAT1, a Novel Serum-Based Biomarker, Enhances Cell Growth by Sponging miR-326 in Oesophageal Squamous Cell Carcinoma.* Cell death & disease 10.7 (2019): 513–14. Web.

133. Guo, Ran et al. *Long Non-Coding RNA PRNCR1 Modulates Non-Small Cell Lung Cancer Cell Proliferation, Apoptosis, Migration, Invasion, and EMT through PRNCR1/miR-126-5p/MTDH Axis.* Bioscience reports 40.7 (2020): n. pag. Web.

134. Xie, Min et al. *Decreased Long Noncoding RNA SPRY4-IT1 Contributing to Gastric Cancer Cell Metastasis Partly via Affecting Epithelial–mesenchymal Transition.*Journal of translational medicine 13.1 (2015): 250–. Web.

135. Jing, F, Zhao, J, Jing, X, Lei, G. *Long noncoding RNA Airn protects podocytes from diabetic nephropathy lesions via binding to Igf2bp2 and facilitating translation of Igf2 and Lamb2*. *Cell Biol Int*. 2020; 44: 1860–1869.

136. Shi, Xiaoqing, Xiaoqin Wang, and Yimin Hua. *LncRNA GACAT1 Promotes Gastric Cancer Cell Growth, Invasion and Migration by Regulating miR-149-Mediated Of ZBTB2 and SP1.*Journal of Cancer 9.20 (2018): 3715–3722. Web.

137. Tantai, Jicheng et al. *Combined Identification of Long Non-Coding RNA XIST and HIF1A-AS1 in Serum as an Effective Screening for Non-Small Cell Lung Cancer.*International journal of clinical and experimental pathology 8.7 (2015): 7887–7895. Print.

Acknowledgments

I thank all the professors, in particular the coordinator of the PhD school, Prof. Silvia Sacchi, all researchers and laboratory staff of the university, who during these three years have taught me a lot both from an educational and human point of view, helping me in my study and research path.

Special thanks go to Prof. Lucia Tettamanti for helping me professionally in the execution of this study and for the numerous teachings received over the years; Prof. Mauro Fasano, for his great help in organizing the results and in the bioinformatics structuring of work; Prof. Alberto Passi and Dr.ssa Paola Moretto for following me in the design and implementation phase of the molecular biology experiments;Dr. Lorenzo Azzi for his availability in patient selection and clinical interpretation of the data during the trial; Prof. Francesco Spadari and Prof. Reinhold J. Medina, for the precious advice they gave me and for the time dedicated to me for the revision of the thesis.

A dutiful thanks to my family, my wife and my children Lorenzo and Giorgia for being close to me and supporting me in achieving this important milestone.