Università
degli Studi
della Campania
Luigi Vanvitelli

DOTTORATO DI RICERCA IN

"SCIENZE BIOCHIMICHE E BIOTECNOLOGICHE"

XXXIV CICLO

SETTORE SCIENTIFICO-DISCIPLINARE BIO/10

Curriculum: Biologia, Biochimica e Biotecnologie Mediche

ANNO ACCADEMICO 2020-2021

Identification of Serum miRNA Signature for Laryngeal Squamous Cell Cancer and in vitro Characterization of miR-223 Functional Role

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RIASSUNTO

Il carcinoma della laringe, classificato come squamocellulare (LSCC) nel 90% dei casi, rappresenta circa un terzo di tutte le neoplasie del distretto testa-collo (Piotrowski I, 2021) e può colpire diversi distretti della laringe, con differenti sintomi ed opzioni di trattamento. Sulla base dei dati raccolti dal Programma SEER (Surveillance, Epidemiology, and End Results), nel periodo 2011-2017, è stata stimata una sopravvivenza specifica per malattia a 5 anni di circa il 60,7%, anche se il tasso risulta essere fortemente correlato allo stadio della malattia alla diagnosi, con elevato grado di successo (80-90%) negli stadi T1 e T2. Consumo di alcol e tabacco ed infezioni da HPV, in particolare del sierotipo HPV16, rappresentano i principali fattori di rischio (Kreimer AR, 2005). Lo screening e la diagnosi precoci garantirebbero la scelta tra un'ampia gamma di potenziali strategie terapeutiche di successo, ma restano ad oggi una sfida a causa dell'assenza di sintomi, di biomarcatori specifici e delle limitazioni insite nelle convenzionali strumentazioni di imaging. Per facilitare quindi la diagnosi e/o l'individuazione di pazienti ad alto rischio di recidiva e migliorare la qualità di vita del paziente affetto da LSCC, si è sempre più indirizzati verso la ricerca di marcatori molecolari affidabili e non invasivi, dotati di alta specificità e sensibilità che permettano di identificare lesioni precoci attraverso lo studio di processi molecolari indicativi di aggressività tumorale, quale ad esempio la transizione epitelio-mesenchimale (EMT), evento cruciale nella progressione tumorale (Yilmaz M, 2009). In questo contesto, di particolare rilevanza sono i marcatori rilasciati nel sangue, nella saliva o nelle urine che eviterebbero l'uso di metodiche invasive per il paziente. Tra questi, i microRNA (miRNA) circolanti nei diversi fluidi biologici (Tie Y, 2009) che, grazie alla stabilità conferita dall'inclusione in microvescicole, esosomi, cellule tumorali circolanti (CTCs) o dal legame a proteine argonauta o lipoproteine ad alta densità, rappresentano una nuova e promettente classe di biomarcatori diagnostici e prognostici. Inoltre, i miRNA agiscono come regolatori del microambiente tumorale, anch'esso implicato nella progressione del tumore (Redis RS, 2012).

Sulla base di tali premesse, lo scopo del mio progetto di dottorato è stato quello di identificare e caratterizzare miRNA sierici la cui espressione fosse deregolata nei pazienti affetti da LSCC ed investigarne i meccanismi biologici e molecolari. In dettaglio, l'analisi in microarray del profilo di espressione di 377 miRNA sierici – condotta su 45 pazienti, di cui 22 con metastasi linfonodali, N^+, e 23 senza coinvolgimento linfonodale, N^-, raffrontati con 23 volontari sani - ha mostrato, tra gli 81 miRNA rilevati, l'up-regolazione di 11 di essi

e la down-regolazione di altri 5. Basandoci sui valori di "fold-change" e sulla relativa significatività statistica, abbiamo focalizzato lo studio sulla validazione dei livelli di espressione, mediante qRT-PCR, di 3 dei miRNA up-regolati (miR-93, miR-223, e miR-532) che sono stati selezionati come possibili biomarcatori. Confermata la significativa up-regolazione su una coorte più ampia di pazienti affetti da LSCC (75 pazienti, divisi in 32 N^+ e 43 N^-), il valore diagnostico di miR-93, miR-223 e miR-532 è stato definito mediante analisi della curva ROC. In particolare, l'area sotto la curva (AUC), ne ha evidenziato un moderato potenziale diagnostico che sarà ulteriormente approfondito durante il progressivo ampliamento della casistica dei pazienti arruolati. Oggetto di studio è stata inoltre l'analisi della correlazione tra i livelli di espressione dei miRNA in oggetto ed alcuni parametri clinico-patologici a nostra disposizione. I risultati, ottenuti calcolando il coefficiente di correlazione *r*, hanno mostrato una debole relazione negativa tra i livelli d'espressione di miR-93 e miR-532 ed il grado del tumore, suggerendone un potenziale diagnostico per gli stadi precoci della malattia. Abbiamo a questo punto deciso di investigare, *in vitro*, il ruolo del miR-223 in un modello cellulare di carcinoma laringeo, analizzando gli effetti molecolari della sua deregolazione. La selezione di tale miRNA è stata incoraggiata dal fatto che, tra i miRNA sierici individuati, fosse l'unico a risultare up-regolato anche a livello tissutale nei pazienti affetti da LSCC, come riportato in un recente studio condotto dal nostro gruppo di ricerca (Ricciardiello F, 2017). In particolare, i dati di espressione tissutale di miR-223 sono stati ripresi al fine di analizzarne la correlazione con l'estensione del tumore, evidenziando una significativa up-regolazione nei pazienti con neoplasia in stadio avanzato (T3-T4) rispetto ai pazienti in stadi più precoci di malattia (T1-T2), un dato che ha suggerito una possibile correlazione tra miR-223 e il volume del tumore. L'analisi bioinformatica dei target predetti di miR-223, condotta mediante l'ausilio di tools di predizione disponibili online, ha permesso di analizzare *in silico* una serie di possibili geni target, tra cui spicca, con uno score di predizione pari a 79, il gene MTSS1 (Metastasis suppressor protein 1) codificante per una proteina oncosoppressiva implicata nel processo metastatico. Per intraprendere lo studio sugli effetti biologici indotti dal miR-223, due linee cellulari di LSCC (HEp-2 e HNO210) sono state trasdotte con vettori lentivirali di terza generazione contenenti i costrutti codificanti per il miR-223 mimic, il miR-223 inhibitor e due sequenze random specifiche che fungessero da controlli negativi. I vettori lentivirali utilizzati contenevano anche un gene codificante per l'antibiotico-resistenza verso la puromicina, indispensabile per la selezione delle cellule infettate, ed un gene reporter GFP utile al monitoraggio dell'efficienza di trasduzione. Quest'ultima è stata valutata sia mediante quantizzazione relativa dell'intensità

di fluorescenza delle cellule trasdotte rispetto a quelle *wild type*, sia mediante qRT-PCR. I risultati hanno mostrato un significativo aumento dei livelli di espressione del miR-223 nelle cellule trasdotte con miR-223 mimic, rispetto al corrispettivo controllo negativo, ed una meno significativa riduzione dei livelli del miR-223 quando le cellule sono state infettate con miR-223 inhibitor. I saggi di vitalità, clonogenicità e wound-healing hanno confermato l'effetto pro-tumorigenico e pro-metastatico del miR-223 mimic rispetto al proprio controllo negativo. Un effetto opposto invece è stato evidenziato nelle cellule trasdotte con miR-223 inhibitor. In dettaglio, l'espressione ectopica dell'oligonucleotide antisenso limitava in maniera significativa la capacità di sopravvivenza e migrazione cellulare.

Sulla base dei dati presenti in letteratura, che indicano un coinvolgimento del miR-223 nel processo di EMT (Ma J, 2015), gli sviluppi futuri del presente progetto, saranno orientati verso la caratterizzazione del profilo d'espressione dei principali geni che prendono parte a tale processo. Inoltre, sono attualmente in corso studi *in vitro* di analisi metabolomica in NMR e "profiling" diretto dell'espressione di oltre 700 geni correlati col cancro mediante tecnologia digitale NanoString. Tali esperimenti saranno volti ad una migliore comprensione dei meccanismi molecolari alla base dell'effetto pro-tumorigenico del miR-223 ed anti-tumorale del suo inhibitor.

ABSTRACT

Laryngeal squamous cell cancer (LSCC) represents one-third of all head and neck malignancies and involves different regions with various symptoms, spreading patterns and treatment options. Early diagnosis, as well as prompt identification of patients with high risk of relapse would ensure a complete disease resolution or, at least, greater chance of therapeutic success. However, this goal remains a challenge due to the absence of specific biomarkers for this neoplasm. In this context, we aimed at characterizing serum miRNA profile of LSCC patients for the identification of new potential molecular diagnostic and prognostic circulating markers endowed with high selectivity and specificity. Preliminary analysis showed 11 up-regulated and 5 down-regulated serum miRNAs in LSCC patients, compared to healthy donors. Based on the fold-change values and their statistical significance, we focused on three overexpressed miRNA candidates (miR-93, miR-223, miR-532), confirming their prominent up-modulation by qRT-PCR validation tests, as well as their diagnostic value by ROC analysis. Moreover, we analysed the possible correlation between miRNA expression levels and clinical-pathological parameters highlighting a weak negative relation between miR-93 and miR-532 and tumor stage, that suggested their diagnostic potential for the early stages of disease. Then we decided to investigate the biological relevance of miR-223 deregulation using a preclinical *in vitro* LSCC model. This choice was encouraged by previous data regarding miR-223 upregulation in LSCC tissues and our recent findings about a significant up-regulation in patients with advanced cancer (T3-T4) compared to patients in earlier stages of the disease (T1-T2), thus suggesting a possible correlation between miR-223 and tumor extension. The *in vitro* study was carried out on two LSCC cell lines (HEp-2 and HNO210) transduced with 3th generation lentiviral vectors containing the constructs encoding for miR-223 mimic, miR-223 inhibitor and two specific random sequences acting as negative controls. The viability, clonogenicity and wound-healing assays showed pro-tumorigenic and pro-metastatic effects following miR-223 upregulation. An opposite result was observed when cells were transduced with miR-223 inhibitor. In detail, the ectopic expression of the antisense oligonucleotide significantly limited cell viability and migration.

Our future studies will be finalized at characterizing the role played by miR-223 in epithelial-mesenchymal transition (EMT) process, as well as at defining the molecular bases of the miR-223-mediated pro-tumorigenic effect availing of metabolomic NMR analysis and direct gene profiling by NanoString digital technology.

CONTENTS

I INTRODUCTION

Laryngeal Squamous Cell Cancer (LSCC) is the sixth most common cancer worldwide. The 5-years relative survival rate for patients diagnosed for LSCC is 60,7%. One of the main factors affecting this index is represented by the high percentage of diagnosis at advanced stage, thus negatively impacting on prognosis and mortality (National Cancer Institute: Surveillance s.d.). Carcinogenesis has been widely investigated in the last years, focusing on genetic and molecular modifications occurring in neoplastic transformation and progression. Moreover, the identification of new diagnostic and prognostic markers is the main challenge to date. Ideally a good diagnostic and/or prognostic factor should provide reliable information on neoplasms' evolution, as well as on its possible response to the various therapeutic options in order to plan the best treatment strategy. Moreover, it should be easily detected, at low cost and with non-invasive techniques. To date, it is possible to obtain prognostic information through the examination of some clinical, pathological and molecular factors, but there is still considerable confusion regarding their meaning and possible applications in the clinical setting. MicroRNAs (miRNAs) are a class of non-coding single-stranded RNAs of 18-22 nucleotides, involved in post-transcriptional regulation of gene expression via base-pairing with the complementary sequences in the 3′ untranslated region (3′UTR) of their target messenger RNAs (mRNAs). They inhibit gene expression by promoting translational repression, mRNA target cleavage or deadenylation (Grimaldi A, 2018). At present, a large number of miRNAs are known to be involved in a variety of normal biological functions but also in tumorigenic events.

Many reports showed that miRNAs are aberrantly regulated in LSCC tissues, according to high-throughput miRNAs microarray analysis (Ricciardiello F, 2017) (Cao P, 2013). Differential miRNA expression pattern was also confirmed in LSCC patients' plasma (Ayaz L, 2013). Some relationships have been revealed between tissue/sera miRNA levels and migration, invasion, metastasis, tumor infiltration, and disease relapse. Additionally, a number of researches have demonstrated the oncogenic or tumor-suppressive role played by miRNAs in LSCC, based on *in vitro* and *in vivo* results. However, the function and the molecular mechanisms in disease onset and metastasization still require to be clarified. Hence, the need to identify specific diagnostic and/or prognostic biomarkers, as well as to recognize their specific biological function, maybe exploiting the anti-tumor and anti-

metastatic potential for therapeutic purpose, has prompted us to carry out the present study.

1.1 Laryngeal Cancer

Laryngeal cancer represents one-third of all head and neck malignancies. Larynx is a part of throat, between the base of the tongue and trachea, that contains the vocal cords, which are involved in sounds emission. The vast majority of laryngeal cancers are well-differentiated squamous cell carcinomas (LSCCs), accounting over 90% of the cases (Almadori G, 2005). A minority of cases represent squamous cell variants, including verrucous carcinoma, sarcomatoid carcinoma, and neuroendocrine carcinoma. The histopathological spectrum of the SCCs varies significantly from hyperplasia, dysplasia and carcinoma *in situ* to invasive cancer and involves different subsites of the larynx, with different implications in symptomatic manifestations, spreading patterns and treatment options. Spreading patterns usually depend on primary mass location and on lymphatic system involvement. The latter is a pathologic hallmark of supraglottic cancers for almost 55% patients. Unless the supraglottic or glottic extension, the vocal cords do not present lymphatic involvement risk. Glottic cancers typically are confined at the anterior portion of the upper free margin of one vocal cord. The subglottis extends superiorly from 5 mm below the free margin of the vocal cord and inferiorly to the lower border of the cricoid cartilage (or 10 mm below the apex of the ventricle). They also have sparse lymphatic supply, with drainage collecting into levels IV and VI of the cervical nodal chain (Ballo MT, 1998) (O'Sullivan B, 1995) (**Figure 1**).

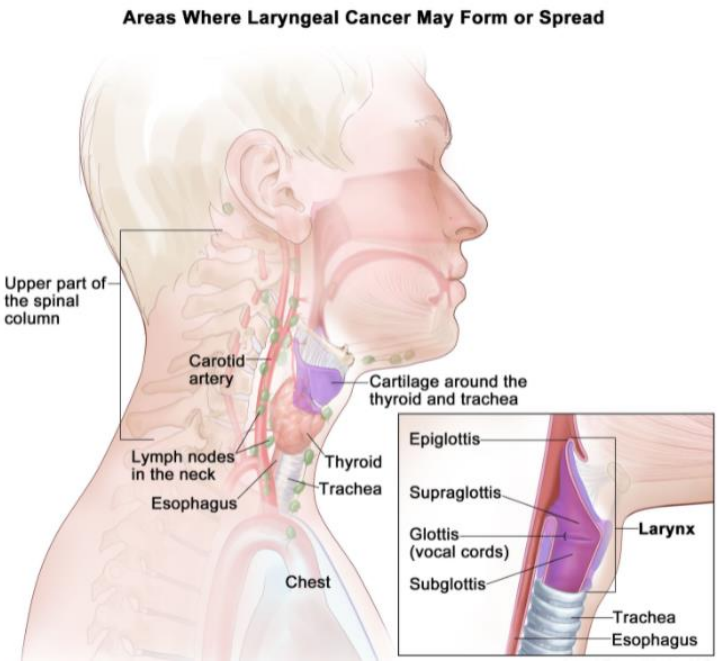


Figure1 Laryngeal Cancer. LSCC occurs in laryngeal tissue (area of the throat that contains the vocal cords). The larynx includes the supraglottis, glottis (vocal cords), and subglottis. The cancer may spread to nearby tissues or to the thyroid, trachea, or esophagus. It may also spread to the neck lymph nodes, the carotid artery, the upper part of the spinal column, the chest, and to other parts of the body (National Cancer Institute: Surveillance s.d.).

During 2020, 184,515 new cases were diagnosed globally, while 99,840 deaths were recorded (Sung H, 2021). Laryngeal cancer becomes more common with age and is more frequent in men than in women. The rate of new cases of laryngeal cancer is 2.8, while the death rate is 0.9 per 100,000 men and women per year (**Figure 2**). Moreover, SEER data report a rate of new cases, sex adjusted, by 4.9 per 100 000 for males and 1.1 for females, whereas the median age at diagnosis is 66 years for both sex (National Cancer Institute: Surveillance s.d.).

Figure 2 Rate of New Cases and Deaths. The rate of new cases of laryngeal cancer was 2.8 per 100,000 men and women per year. The death rate was 0.9 per 100,000 men and women per year. These rates are age-adjusted and based on 2014–2018 cases and 2015–2019 deaths (National Cancer Institute: Surveillance s.d.).

Based on the data coming from the Surveillance, Epidemiology, and End Results (SEER) Program, from 2011 to 2017, the estimated 5-years relative survival for patients diagnosed for laryngeal cancer was 60,7% as also found in the last decades (National Cancer Institute: Surveillance s.d.). One of the main factors affecting this index is represented by the high percentage of diagnosis at advanced stage, approximately 60% (Groome PA, 2003). In fact, the 5-years relative survival for localized laryngeal cancer increases up to 77.9% (National Cancer Institute: Surveillance s.d.) (**Figure 3**). Additionally, it was demonstrated that the incidence and prevalence have increased by 12.0% and 23.8% during past three decades, individually. On the other hand, the mortality has declined by approximately 5.0% during the term. Among all types of malignancy, LSCC was ranked 22nd in incidence (0.89% of all cancers) and 18th in prevalence (1.44% of all cancers) and deaths (1.39% of all cancer deaths) (Nocini R, 2020).

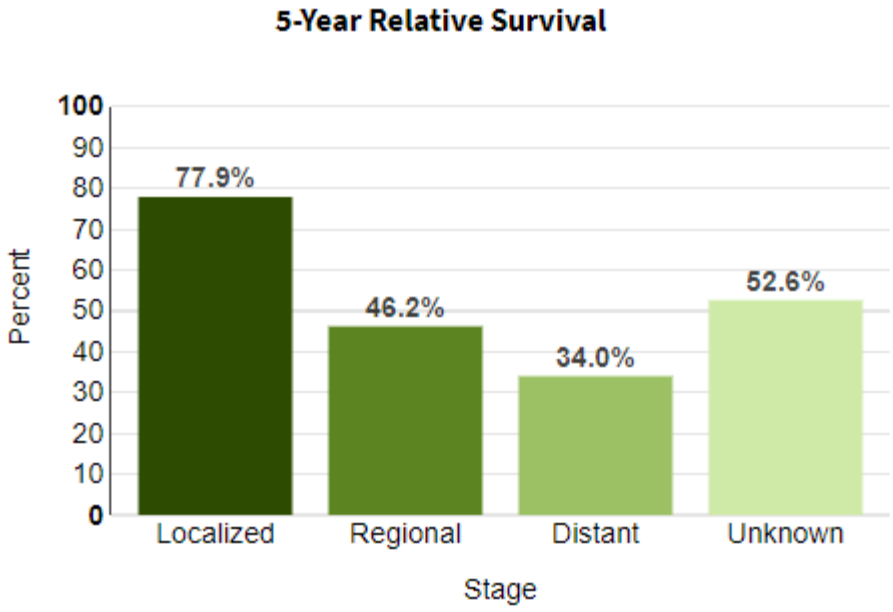


Figure 3 5-Year Relative Survival by Stage at Diagnosis. Cancer stage at diagnosis has a strong influence on survival. A cancer confined to primary site is defined *localized*; if it spreads to a different part of the body, it is *regional* or *distant*. *Unknown* refers to unstaged cancers (National Cancer Institute: Surveillance s.d.).

Tabacco and alcohol consumption are the most important risk factors in LSCC pathogenesis. It was reported that they can affect with different incidences various anatomical sites of the larynx: tabacco is mostly associated with glottis and vocal cord cancer, while abuse of alcoholic beverages constitutes a greater risk for tumor development in supraglottic region (Muscat JE, 1992). Moreover, it has been demonstrated that alcohol and tobacco have a multiplicative effect on LSCC risk (Bosetti C, 2002). In recent years, reduction smoking rates has led to a downturn in both incidence and mortality.

Dietary has been known as another important risk factor for LSCC (Garavello W, 2009) (Di Maso M, 2013) (Maasland DH, 2015). A diet rich in fruit and vegetables, or the intake of vitamins such as carotenoids, retinol and vitamin C, can reduce the risk of developing laryngeal cancer (Glade MJ, 1999); instead, daily consumption of red meat significantly increases the risk for LSCC (OR=1.46, 95% CI: 1.30-1.64) (Di Maso M, 2013). Environmental pollution, HPV infection, gastroesophageal reflux disease or exposure to asbestos are classified as additional risk factors (**Figure 4**). A correlation has been observed in workers exposed to asbestos, wood and textile dusts, polycyclic aromatic hydrocarbons (Smith AH, 1990) (Stell PM, 1973) (Paget-Bailly S, 2012) (Wynder EL, 1976) (Brown LM,

1988).

Human papillomavirus (HPV), especially HPV16 and HPV18 types, has been recognized as a major risk factor for head and neck cancer (Syrjänen S, 2005) (Kreimer AR, 2005). Hernandez *et al*, in their study, detected HPV in approximately 21% of LSCC patients (31/148 cases) with the most common types HPV16 and HPV33 (Kreimer AR, 2005). In addition, the HPV-positive ratio was higher in female than in male (OR=2.84, 95%CI: 1.07-7.51) (Hernandez BY, 2014).

However, LSCC has a multifactorial aetiology, which consists into exposure to environmental factors (smoking, alcohol, vocal abuse, inhalation of dust or gas, etc) associated with genetic predisposition. In fact, although the neoplasm is rarely associated with hereditary syndromes or tumors, a high incidence of head and neck neoplasms has been found in subjects suffering from type 2 neurofibromatosis (NF2 gene) (Naunheim MR, 2016), Lynch syndrome (p53) (Lynch HT, 1988), Fanconi anemia (FANCF gene) and BRCA2 gene mutations (Szaumkessel M, 2011).

Figure 4 Laryngeal Cancer Aetiology. Most common risk factors include environmental factors, gastroesophageal reflux, viral infections, diet, tobacco and alcohol consumption.

The first step in LSCC diagnosis is represented by the physical exam of the throat, check of the neck for the occurrence of lymph nodes and interview on patient's habitudes, past diseases and medical treatments. When the physical examination indicates a suspect for abnormal areas, the next step is biopsy. During direct nasendoscopy and laryngoscopy the suspected primary lesion is visualized. Cells or tissues are removed for a subsequent observation in microscopy by a pathologist to check for signs of cancer.

There are two methods of biopsy: endoscopic biopsy and fine needle aspiration (FNA) biopsy. Endoscopic biopsy is conducted under general anaesthesia during laryngoscopy, a special equipment is attached to the endoscope and removes tissue samples from larynx. On the other hand, FNA biopsy is used to collect a tissue sample from the tumor region using a needle passing through skin into target position. In addition, FNA biopsy is also performed to confirm the presence of cancer spread in lymph nodes. Conventional imaging techniques are useful to confirm tumor mass presence, but they are particularly important to investigate size, location and extension of malignancy. For all laryngeal cancers, whether suspected to be early or late stage, imaging of the primary lesion and draining lymph nodes is indicated, usually with contrast-enhanced CT of the neck. This study visualizes the neck lymphatics, as well as structures that cannot be assessed adequately even with direct laryngoscopy, such as the subglottic region, as well as to detect subtle signs of disease extension such as minor invasion into the thyroid cartilage, all of which are crucial for accurate staging. MRI (magnetic resonance imaging), PET scan (positron emission tomography scan), PET-CT scan**,** Bone scan and Barium swallow can be requested to investigate the suspected locally advanced disease, or to rule out distant metastases and esophago-gastro-duodeno involvement (Marioni G, 2006). All these approaches for LSCC detection and stage definition are potentially invasive. Thus, reliable minimally-invasive diagnostic techniques for disease detection and monitoring are largely desired.

Tumor stage is a prominent factor for treatment planning. LSCC clinical stage is defined by TNM classification system, a global standard for cancer progression classification. The "T" describes the size of primary tumor, and the presence or absence of invasion nearby the tissue. The "N" explains presence or absence of regional lymph node metastasis. The "M" shows presence or absence of distant metastasis, *i.e.* cancer spreading from primary site to different site within the body (**Figure 5**). If the cancer is removed by surgery, cancer tissue will be examined by a pathologist under a microscope. Sometimes, the pathologist's review will result in clinical TNM (cTNM) changing into pathological TNM (pTNM), thus determining also a change in the therapeutic indications (Marioni G, 2006).

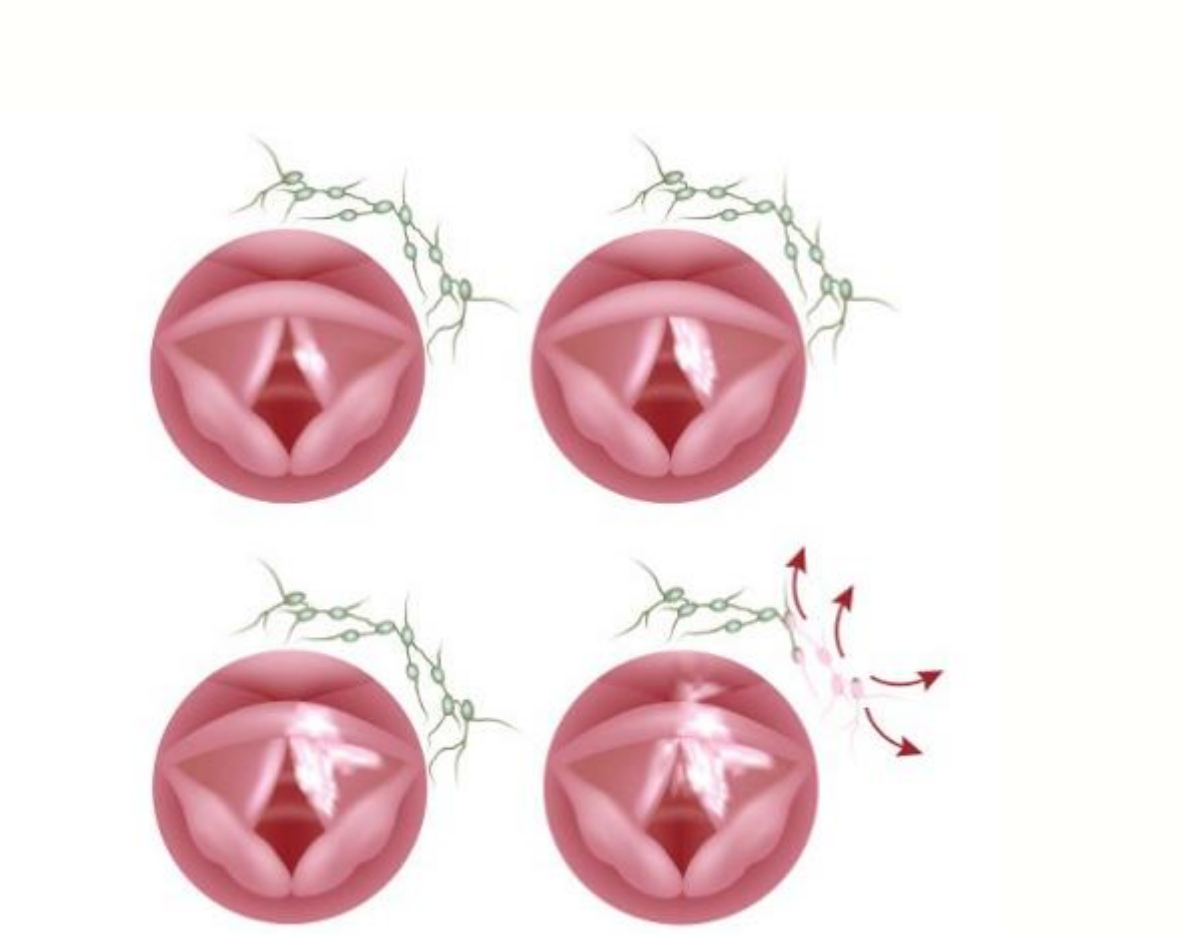


Figure 5 Stages of Laryngeal Cancer. Stage I: tumor is localized in a single part of the larynx, Stage II: tumor is localized in more than one part, Stage III: tumor is throughout but has not spread outside the larynx, Stage IV: tumor has grown into body tissues outside the larynx.

In the past laryngeal cancer treatment was purely surgical, but the current treatment approach is directed towards organ preservation by chemoradiation. Many studies show that this approach produces similar results to total laryngectomy. However, treatment options mainly depend on tumor stage, location and size. Early-stage disease is highly curable with either surgical or radiation monotherapy, often larynx-preserving, whereas late-stage disease has a worse outcome, warrants multimodal therapy, and is less often larynx-preserving. For patients requiring laryngectomy, speech rehabilitation methods have improved in the modern era (Mourad M, 2017).

Surgery is a common treatment for all stages of laryngeal cancer. Different surgical procedures, more or less invasive, can be used: Cordectomy, Supraglottic laryngectomy,

Hemilaryngectomy (saves the voice), Partial laryngectomy, Total laryngectomy, Thyroidectomy up to the most innovative Laser surgery that uses a laser beam (a narrow beam of intense light) as a knife to make bloodless cuts in tissue or to remove a surface lesion.

After removing cancer mass, some patients may be given chemotherapy or radiation therapy in order to kill any remaining cancer cells. This type of treatment, called adjuvant therapy, can decrease the risk for cancer relapse. Radiation therapy is useful to kill cancer cells or inhibit cancer cell growth through high energy X-rays or other types of radiation. There are two methods: external and internal radiotherapy. For external radiotherapy, a radiation machine is used to send radiation toward the cancer from outside of patient's body. On the other hand, for internal radiotherapy, radioactive substance is directly placed into or near the tumor using needles, wires or catheters. Hyperfractionated is a particular type of radiotherapy where a smaller than usual total daily dose of radiation is divided into two doses and the treatments are given twice a day. Chemotherapy is, instead, a systemic or regional cancer treatment that uses drugs to stop cancer cell growth, both by killing cells and by stopping cell proliferation. In systemic chemotherapy, chemotherapeutic drugs are incorporated into the bloodstream after oral administration or injection into a vein or muscle, and reach the cancer cells throughout the patient's body. Instead, in regional chemotherapy, the drug is directly placed into cerebrospinal fluid, body cavity or organ, and mainly impacts on cancer cells in those regions (Agra IM, 2012).

The combination of Cisplatin (an inhibitor of DNA replication), 5-fluorouracil (an inhibitor of DNA synthesis) and Docetaxel (an inhibitor of microtubule function) has been used as neo-adjuvant chemotherapy in patients suffering from various head and neck cancers including LSCC. The administration has the aim to achieve a good clinical outcome following definitive treatments, such as surgery and radiotherapy and/or to avoid surgery, performing radiotherapy, as possible, for laryngeal function preservation (Matoba T, 2017). Cisplatin is administered also in combination with radiotherapy to exert powerful anti-tumor effect in order to preserve the larynx and improve patient's quality of life (Bonomi MR, 2018). Among the opportunities offered by biological LSCC therapy, immunotherapy avails of patient's immune system to fight cancer. Nivolumab and Pembrolizumab are PD-1 inhibitors used to treat metastatic or recurrent laryngeal cancer (Cohen EEW, 2019). PD-1 is a protein located on T cell surface and acts as immune checkpoint that provides inhibitory signals to attenuate T cell activity. PD-L1 is a protein found on various types of cancer cells. When PD-1 binds to PD-L1, it stops the T cell from killing the cancer cell. So, PD-1 and

PD-L1 inhibitors prevent PD-1 and PD-L1 interaction, allowing cancer cell killing by T cells.

In the recent years, the advent of targeted therapy has allowed to reduce the side effects resulting from radiotherapy and chemotherapy. Among these drugs, monoclonal antibodies are used because of their ability to recognize specific targets on cancer cells, suppressing their proliferation. Cetuximab, a mouse/human chimeric monoclonal antibody, has been explored for laryngeal cancer treatment. It targets epidermal growth factor receptor (EGFR) and inhibits cancer cell growth suppressing its function. Cetuximab is usually used in combination with radiotherapy or chemotherapy. It was reported that the combination between Cetuximab and radiotherapy significantly improves LSCC patients' prognosis, including laryngeal preservation rates and laryngectomy-free survival, compared to mere radiotherapy (Bonner J, 2016).

After treatment, laryngeal cancer patients, undergo frequent and careful follow-up. The cancer may relapse in the larynx or in other parts of the body, such as lungs, liver, or bone. Most likely it come back in the first 2 to 3 years. The above said conventional methods such as surgery, radiotherapy and chemotherapy are the gold standards for LSCC treatment, but they could negatively impact on patient's quality of life. In addition, although targeted therapy has attracted a lot of interests in LSCC treatment, the number of practical approaches is still few. Thus, novel therapeutic approaches yielding better results and lower side effects are coveted.

1.2 Non-Coding RNA

Transcription is a complex event in multicellular eukaryotes (Carninci P, 2005). Recent high-throughput transcriptomic analyses allowed to study whole set of RNAs expressed in specific conditions and cell types, revealing a strong modulation depending on the environment or cell development. The transcriptome includes mRNA, tRNA, rRNA and non-coding RNA (ncRNA). The genomic DNA is transcribed for up 90%, while only 1-2 % of transcripts encode for proteins. So, non-protein coding DNA is the most abundant part of genome (Ling H, 2017). The function of these transcripts was largely unknown and considered "junk DNA" for several years. Nowadays, their involvement in important biological functions, as development and/or homeostasis, is increasingly studied (Morris KV, 2014) (Holoch D, 2015). The ncRNAs are a heterogeneous class of RNA molecules

transcribed from non-(protein)-coding regions, which lack an open reading frame and consequently have no apparent protein coding ability. They can be divided into two different families based on their nature: long ncRNA (lncRNA) and small ncRNA (sncRNA). Long non-coding intergenic RNAs and antisense transcripts are included in the first class; snoRNAs (small nucleolar RNA), siRNAs (Small Interfering RNA) and miRNAs (MicroRNAs) belong to the second class. These transcripts play a role in alternative splicing, maturation of rRNAs, tRNAs and other nuclear RNAs (Bachellerie JP, 2002), and are involved in gene silencing through specific base pairing with target molecules. Moreover, they regulate gene expression in both physiological and pathological conditions, from transcription and processing of messenger RNA (mRNA) to translation and correct compartmentalization of proteins, allowing cells to carry out many different functions that sustain cell growth, cellular development and differentiation, apoptosis or other different responses to specific stimuli (Mercer TR, 2013) (Yamamura S, 2018) (**Figure 6**). Emerging evidences from the last two decades have unambiguously proved the functional importance of ncRNAs in human biology and diseases including cancer, and today they represent the main engine for the design of innovative platforms to finalize their pharmacological application as either therapeutic tools or molecular biomarkers (Grimaldi A, 2018).

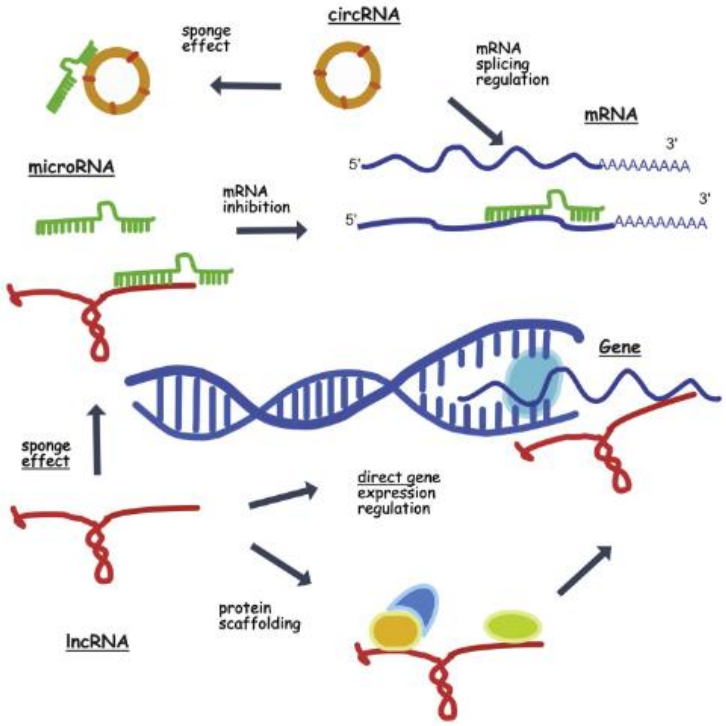


Figure 6 Non-Coding RNAs in Gene Expression Control. ncRNAs are involved in various physiological processes through a variety of RNA molecules that regulate transcription at different levels. microRNAs (green) and lncRNAs (red) can directly regulate gene expression by binding to mRNA (microRNAs), to gene/nascent transcript (lncRNAs) or to histone modifiers bringing them to a gene (lncRNAs). lncRNAs and microRNAs can also regulate each other through the "sponge" effect mediated by CircRNAs (orange circle) (Panni S, 2020).

1.2.1 MicroRNA

MicroRNA (miRNA) is a family of evolutionarily conserved non-coding small RNA molecules, typically 18-22 nucleotides in length, involved in gene expression control (Bartel DP, 2004) (Rana TM, 2007). miRNAs were first discovered in *Caenorhabditis elegans* and subsequently in the majority of eukaryotes, including humans (Perron MP, 2008) (Wightman B, 1993). There are currently 2588 annotated miRNAs in the human genome (Griffiths-Jones S, 2006); about 1-2% of the human genome encodes miRNAs and, based on computational prediction analyses, it has been estimated that approximately 60% of human protein-coding genes are direct miRNA targets through complementary base pairing between the 3 'UTR of messenger RNA and the 5' region of miRNA, called "seed region" (Friedman RC, 2009).

This pairing causes translation inhibition and/or target mRNA degradation (Bartel DP, 2004) (Rajewsky N, 2006) (Rajewsky N, 2006) (Berezikov E, 2005). Most of the miRNAs are localized in introns of protein-coding genes or are transcribed in polycistronic RNA. However, they can also be found in several other genomic regions including exons and introns of non-coding genes (Rodriguez A, 2004), and also in 3' untranslated regions of protein-coding genes (3' UTR) (Cai X, 2004). The temporal and tissue-specific expression of miRNAs suggests that they play an important role in various biological processes, such as embryonic development, cell differentiation, apoptosis, cell growth control and metabolic processes regulation (He L, 2004), through target genes silencing. It is therefore not surprising that miRNA levels are altered in several diseases, primarily tumorigenesis and metastatic progression (Blenkiron C, 2007) (Zhang W, 2007) (Ma L, 2008). miRNA expression profile in cancer suggests that these molecules can act as either oncogenes or tumor suppressors playing crucial roles in carcinogenesis (Chen Y, 2014) (Di Leva G, 2014).

1.2.2 MicroRNA: Biogenesis and Mechanism of Action

miRNA biogenesis can be divided into three phases: transcription and processing into the nucleus, nucleus-cytosol translocation and cytoplasm maturation (Winter J, 2009). Nuclear transcription, mediated by either RNA Polymerase II (Pol II) or RNA polymerase III (Pol III), generates the primary-miRNA (pri-miRNA). This transcript, several hundred nucleotides in length, contains a hairpin stem of 33 base-pairs, a terminal loop and two single-stranded flanking regions upstream and downstream of the hairpin (Lee Y, 2004) (Borchert GM, 2006). Each pri-miRNA can produce either a single miRNA or more than one if miRNA genes are organized in clusters that are processed from a common primary transcript, known as polycistronic. A typical monocistronic pri-miRNA contains a cap of 7-methylguanosine at the 5’-end and a poly (A) tail at the 3’-end (Köhler A, 2007) (Liu X, 2008). In the following step, a miRNA precursor (pre-miRNA), is produced through the action of the nuclear Microprocessor complex. The core of this complex is represented by the DROSHA enzyme, a ribonuclease (RNase) III with endonuclease activity, which acts in cooperation with the essential co-factor double-stranded RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8). DROSHA contains two RNase III domains (RNase IIIa and RNase IIIb), and each one cleaves one strand of the double-strand RNA towards the base of stem-loop secondary structure contained within pri-miRNA, liberating a hairpin-

shaped 60-70 nucleotide precursor miRNA (pre-miRNA) (Gregory RI, 2004). Then, pre-miRNA is exported from nucleus to cytoplasm by XPO5 and its co-factor RanGTP, that prevent nuclear degradation and facilitate translocation (Yi R, 2003) (Lund E, 2004). In the cytoplasm, the hairpin precursor is further cleaved by DICER1, a multi-domain protein with an N-terminal ATPase/Helicase domain, a DUF283, Piwi/Argonaute/Zwilli (PAZ) domain, and two tandem RNase III nuclease domains (RNase IIIa and RNase IIIb) located at the C-terminal followed by a double-stranded RNA-binding domain (dsRBD) (Ma E, 2012). DICER1, acting in complex with transactivation responsive RNA-binding protein (TRBP), cleaves the hairpin pre-miRNA close to the terminal loop sequence and finally produces a roughly 22-nucleotide miRNA duplex with two nucleotides as overhangs at each 3'-end. On the base of the thermodynamic stability of the base pairs at the two ends of the miRNA duplex, two different strands can theoretically be generated: i) *guide strand*, with the less stable base pair at its 5'-end, able to induce gene silencing; ii) *passenger strand* that will be degraded (Khvorova A, 2003). miRNA-induced silencing complex (miRISC) is the effector cytoplasmic machine in which TBRP connects DICER1 with Argonaute proteins (AGO1-4), promoting their assembly (Chendrimada TP, 2005). In miRISC, the mature miRNA binds AGO2 and, together with members of the GW182 family proteins, guides the post-transcriptional regulation of gene expression in cytoplasmic processing bodies (P-bodies). P-bodies are cytoplasmic ribonucleoprotein (RNP) granules primarily composed of translationally repressed mRNAs and a variety of enzymes and factors related to mRNA decay, suggesting roles in post-transcriptional regulation (Luo Y, 2018). Here, a sequence of 6-7 nucleotide near the seed sequence binds to the 3′UTR of target mRNAs with an imperfect but efficient paring (Lin S, 2015) (**Figure 7**).

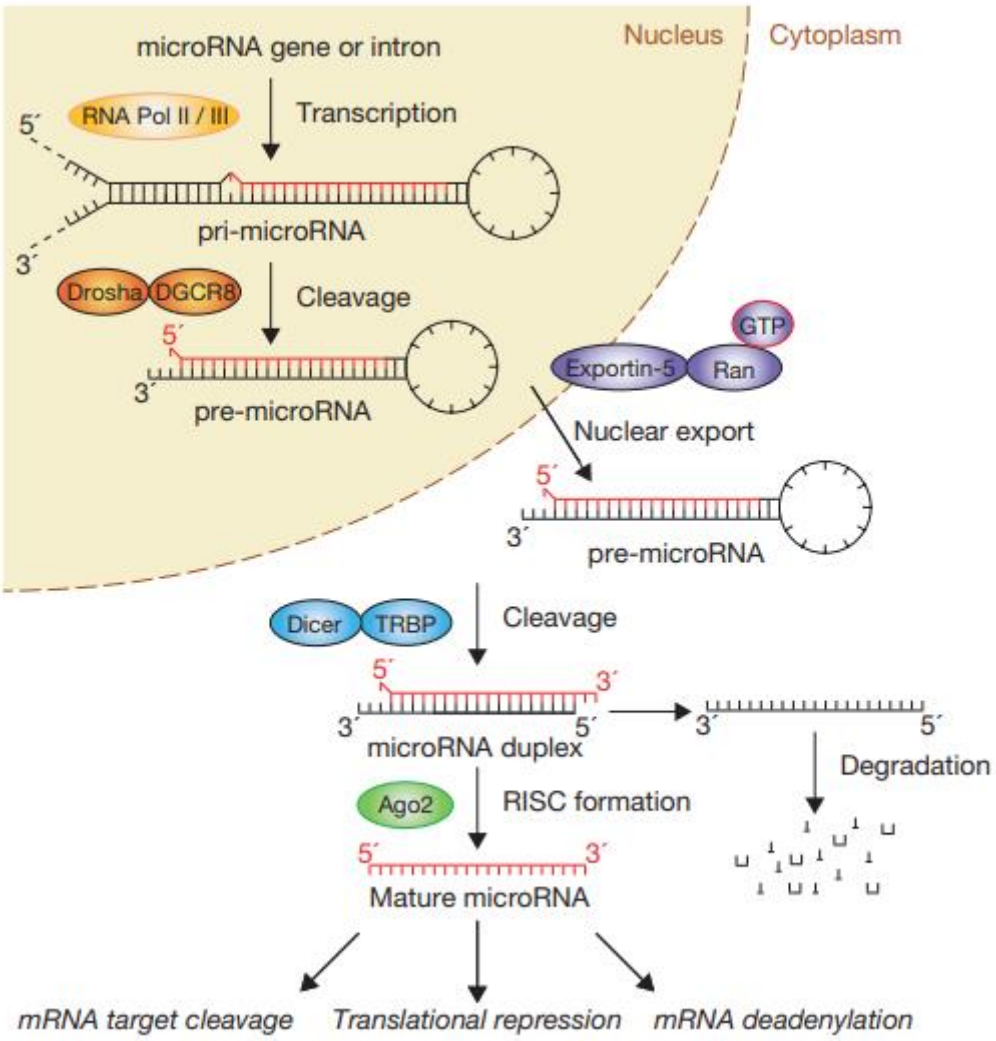


Figure 7 Canonical Pathway of microRNA Processing. This canonical maturation includes the production of the pri-miRNA transcript by RNA polymerase II or III and pri-miRNA cleavage by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer complexed with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. (Winter J, 2009).

1.2.3 MicroRNA and Cancer: Biomarker, Cellular Function and Therapeutic Application

In the previous two decades, it has been widely shown that miRNA dysregulation is associated with the pathogenesis of many human diseases, including cancer. In particular, aberrantly expressed miRNAs in human malignancies play a critical role in both cancer initiation and progression (Di Leva G, 2014). The first work highlighting a link between miRNAs and cancer, was carried out on human leukemias, specifically chronic lymphocytic leukemia (CLL). About 50% of CLL cases have a deletion in the chromosomal region 13q14. This deletion, usually hemizygous, can sometimes involve both chromosomes, resulting in homozygous deletions. The characterization of a small region of about 30kb, commonly subject to homozygous deletions, has highlighted the presence of two genes coding for microRNA, *i.e.* miR15a and miR16-1. Expression analysis demonstrated that these two genes are negatively modulated in over 68% of CLLs, suggesting the involvement of this loss in CLL pathogenesis. Later, it was observed that mutations in the primary precursors of both miRNAs reduced their expression, causing the overexpression of their target gene BCL-2, an anti-apoptotic gene frequently upregulated in CLL (Calin GA, 2002) (Calin GA, 2004) (Cimmino A, 2005). To date, a growing body of evidence has demonstrated different mechanisms, including chromosomal abnormalities, transcriptional control changes, epigenetic alterations and defects in miRNA biogenesis machinery as a possible cause of miRNAs expression dysregulation (Iorio MV, 2012).

The genomic analysis of miRNA genetic sequences revealed a correlation between epigenetic events occurring in human cancers, such as hypermetilation of CpG islands in gene promoters, disruption of the histone modification patterns, and global genomic DNA hypomethylation (Liu X, 2013). Likewise, mutations or aberrant expression of any components of the miRNA biogenesis machinery could lead to aberrant expression of miRNAs. For example, the increased expression of DROSHA alters the global miRNA expression profile and promotes cell proliferation, migration and invasion of neoplastic cells (Muralidhar B, 2011). Conversely, DROSHA downregulation in many other types of cancer results in decreased miRNA expression and is correlated with metastasis, invasion and poor patient survival (Kumar MS, 2007). Microsatellite instability induced by XPO5 inactivating mutations in sporadic colon, gastric and endometrial cancers, promotes accumulation of pre-miRNA in the nucleus, inhibiting its cytoplasmic translocation with the subsequent alteration of miRNA biogenesis (Melo SA, 2010). Instead, DICER1 mutations are frequently found in

different types of inherited tumors predisposing to the DICER1 syndrome, in which DICER1 expression decrease and/or function impairment, promotes the abnormal expression of miRNAs and cancer pathogenesis (Slade I, 2011) (Torrezan GT, 2014).

miRNAs' genomic location is not random, since the most frequent location is in fragile sites of chromosomes and in chromosomal regions already mapped for the presence of a tumor (Dalmay T, 2008).

These findings, together with recent bioinformatics and experimental studies which state that 60% of human genes are direct targets for miRNAs, suggest their role in almost all biological processes including cell cycle regulation, proliferation, cell growth, apoptosis, invasion, migration, angiogenesis, differentiation and stress responses. Depending on their target genes, miRNAs could function as either tumor suppressor or oncogene under certain conditions (Hayes J, 2014) (**Figure 8**). Downregulated miRNAs, that normally prevent tumor development by inhibiting specific oncogenes and/or genes involved in apoptosis or in the control of cell differentiation, are known as tumor suppressor miRNAs, while miRNAs up-regulated in cancer cells can contribute to carcinogenesis by inhibiting tumor suppressor genes and are considered oncogenic miRNAs (oncomiRs) (Esquela-Kerscher A, 2006).

A clear example of miRNAs acting as tumor suppressors is given, apart from the aforementioned miR-15a and miR-16–1, by miRNA let-7. It is a very conserved gene and, in humans, it is located in a chromosomal region often deleted in cancer (Boyerinas B, 2010). Takamizawa and collaborators found that let-7 is weakly expressed in lung cancer cells and this decrease is significantly associated with shorter postoperative patient survival (Takamizawa J, 2004). More recent studies have identified the RAS oncogene as one of the let-7 targets; in normal conditions let-7 negatively regulates RAS, while in lung cancer let-7 is significantly under-expressed and RAS levels increase confirming the tumor suppressor function of this gene (Johnson SM, 2005). MiR-34a, a member of miR-34 family, is another well characterized tumor suppressor miRNA in a variety of tumors, including breast cancer, prostate cancer, multiple myeloma (MM), lung cancer, liver cancer, colorectal cancer, osteosarcoma, acute myeloid leukemia (AML) and CLL (Misso G, 2014) (Zhang L, 2019). In a positive feedback loop, p53 can induce miR-34a to trigger apoptosis through direct binding to mir-34a promoter. In turn, miR-34a promotes p53 expression by targeting SIRT1, a negative regulator of p53 via deacetylation, increasing p53 tumor suppressor activity (Yamakuchi M, 2009). In fact, similar to p53-mediated phenotypes, miR-34a promotes cell-cycle arrest, cell senescence and apoptosis in cancer cells targeting c-Myc, E2F, CDK4, CDK6, Bcl-2, SIRT1 and c-Met (Misso G, 2014) (Hermeking H, 2010). The first example

of oncogenic miRNA was miR-155 in paediatric Burkitt lymphoma, which derives from the primary transcript known as B-cell integration cluster (BIC) (Metzler M, 2004). The overexpression of miR-155 leads to leukemogenesis through down-regulation of SHIP1 and disruption of B-cell receptor signaling in CLL (Cui B, 2014). Moreover, aberrant expression of miR-155 was reported in Hodgkin lymphoma and in primary mediastinal and diffuse Large-B-cell lymphomas, as well as in solid tumors such as breast cancer (Eis PS, 2005) (Kluiver J, 2005) (Kim S, 2018). The miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b–1, miR-20a and miR-92–1) is a polycistronic miRNA located on chromosome 13q31, a gene locus that is amplified in lung cancer and in a number of lymphomas. This cluster has oncogenic functions and its members were found significantly over-expressed in many solid tumors and haematological malignancies, particularly in their more aggressive forms (Hayashita Y, 2005). In addition, the miR-17-92 cluster was shown to promote proliferation, angiogenesis, and cell survival, regulating E2F, PTEN, p21 and BIM (Fuziwara CS, 2015). MiRNAs can also be involved in the different steps of the metastatic process that finally imply tumor cell translocation from primary site to distant organs: EMT, degradation of extracellular matrix, intravasation, circulating via blood stream or lymph fluids and extravasation (Chan SH, 2015). To date, many reports have demonstrated the role of miR-10b, which promotes metastatic factors such as cell invasion, migration and proliferation in various types of tumor, including breast cancer, colorectal cancer, nasopharyngeal carcinoma, endometrial cancer, pancreatic cancer, hepatic cancer, lung cancer, gastric cancer, esophageal cancer, osteosarcoma, bladder cancer and laryngeal cancer (Sheedy P, 2018). In hepatocellular carcinoma, it has been reported that miR-10b promotes metastasis directly targeting cell adhesion molecule 2 (CADM2) (Li D, 2018). Instead, in osteosarcoma cell progression and invasion was increased via direct targeting of Kruppel-like factor 4 (KLF4), a tumor-suppressor involved in several cancers including colorectal, esophageal and bladder cancers (Wang J, 2016). Moreover, it has been observed that the alterations of metastasis factors by miR-10b aggravate clinical outcomes impacting on tumor size, clinical stage and relapse-free survival rate (Sheedy P, 2018).

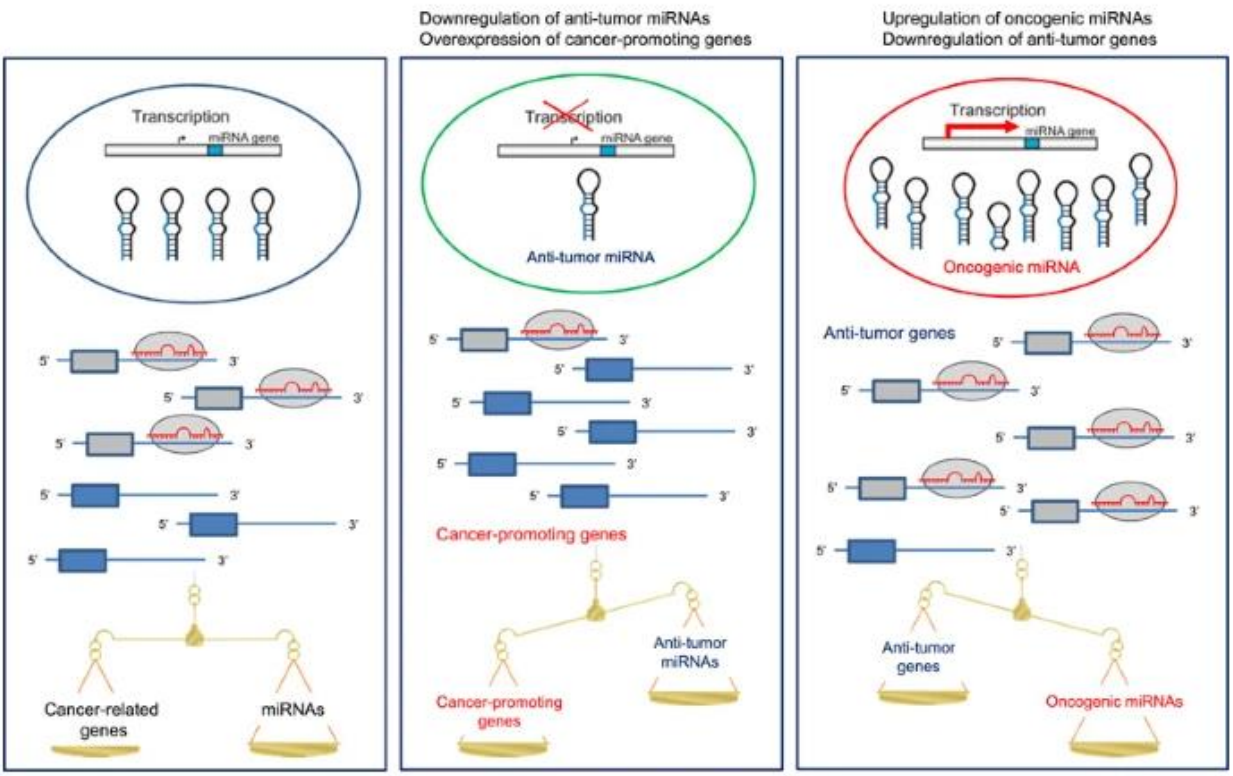


Figure 8 OncomiRs and Tumor-suppressive miRNAs in Cancer Cells. miRNAs can be classified into two main classes: overexpressed miRNAs can act as oncogenes by repressing tumor suppressor genes, while underexpressed miRNA may function as antitumor miRNA by negatively regulating cancer-promoting genes. (Koshizuka K, 2017).

Today it is clear the usefulness of exploiting miRNA expression profiles as molecular tools in tumor diagnosis, prognosis, and therapeutic monitoring. To date, many studies aimed to delineate a stable and unique miRNA signature for each specific tumor type, to distinguish tumors from normal tissues and to sub-classify tumors based on specific clinic-pathological features also predicting patient outcome (Berindan-Neagoe I, 2014).

Regarding the opportunity to develop novel miRNA-based therapeutic strategies, in the light of all the advancements, two therapeutic approaches have been hypothesized: blocking oncomiRs or increasing tumor suppressor miRNAs, with the use of antisense oligonucleotides (ASOs) or miRNA mimic, respectively. Since oncomiRs are often over-expressed in various cancers, their inhibition could restore the function of their target genes responsible for suppressing tumor progression. Several miRNA inhibitory agents have already been tested in preclinical and clinical studies; they include antimiRs consisting in

antisense oligonucleotides (Garzon R, 2010), antagomiRs consisting of locked nucleic acids (Vester B, 2004), miRNA-sponge (Ebert MS, 2007), miRNA-mask (Choi WY, 2007) and small molecules capable of inhibiting miRNA function (Gumireddy K, 2008).

Theoretically, anti-miRNA oligonucleotides (AMOs) can be used to suppress the activity of a specific miRNAs if binding is strong and remains stable under different physiological conditions (Krützfeldt J, 2007). DNA and RNA ONs have a very low stability in serum due to rapid blood clearance (half-life of about 5 min); moreover, due to the polyanionic and macromolecular nature, these molecules cannot diffuse through the cell membrane, with consequent poor intracellular uptake (De Rosa G, 2009) (Farooqi AA, 2013) (Zhang Y, 2013).

Chemical modifications of ONs, such as 2'-O-methylated RNA nucleotides and locked nucleotides (LNA), as well as the use of delivery systems, *i.e.* viral and non-viral vectors, have been investigated to increase their biological stability and to enhance cell uptake. These modifications are also used in the opposite approach where the modified oligonucleotides are released in cells that under-express miRNAs with tumor suppressor activity.

Efficient delivery of miRNA has been achieved with viral vectors, such as Adenovirus, Lentivirus, Herpes Simplex virus; however, the high toxicity, the risk to produce an immune response, the very high cost of the therapy and the difficulties to shift toward the industrial production, hampered a large scale use of these vectors (Thomas CE, 2003) (Tomanin R, 2004). On the contrary, non-viral vectors are more safe and cheaper, although these systems need optimized formulations to obtain high delivery efficiency. The encapsulation of ONs in nanotechnology-based non-viral vectors (lipid-; polymers-; inorganic-nanovectors) allows to protect the nucleic acid toward enzymatic degradation and to prolong the systemic circulation time following intravenous administration. To increase the specificity of drug delivery, it is possible to “decorate” the surface of nanovectors with specific ligands able to bind over-expressed receptors on the target cells (Uchino K, 2013). Different ligands, *i.e.* proteins, oligosaccharides or antibodies, have been proposed for targeted delivery of miRNAs in different tumor cells (Uchino K, 2013). For example, transferrin is one of the most investigated ligand for active targeting with nanovectors, due to its receptor overexpression in numerous cancer cells. Among the non-viral approaches, lipid-nanocarriers are certainly the most investigated delivery systems. Lipid nanovectors are generally biodegradable and biocompatible, since they can contain lipids already present in human cells (Wang X, 2013). Vesicles based on ionizable lipids developed for nucleic acids’ delivery could be considered an evolution of cationic liposomes. These vesicles have also

been defined as stable nucleic acid lipid particles (SNALPs). Liposomes are composed of ionizable cationic lipid 1,2-dioleyl-3-dimethylammonium propane (DODAP) and a PEG moiety which is on the surface of the vesicles. The presence of a positive charge ensures high encapsulation efficiency of nucleic acid. Then, the presence of PEG on the vesicle surface and the possibility to neutralize the surface charge led to a high stability in blood and prolonged circulation time (Semple SC, 2001) (De Rosa G, 2010). SNALPs have been proposed to deliver miRNA for the treatment of different diseases *i.e.* multiple myeloma and medulloblastoma (Zimmermann TS, 2006) (Semple SC, 2010). Finally, ligands, such as transferrin, can be used to improve the specificity for cancer cells of lipid delivery systems (Uchino K, 2013). For this purpose, our research group demonstrated that transferrin decorated SNALPs encapsulating 2'-O-Methylated-miR-34a led high increase of mice survival, compared with untargeted SNALPs in experimental model of multiple myeloma (Scognamiglio I, 2014). Moreover, the chemical modification induced further increase of the mice survival compared to unmodified miR-34a mimic. The biggest challenge for either miRNA mimic- or antisense oligonucleotide-based approaches is the direct injection of modified oligonucleotides into tumor tissues (Vester B, 2004). This strategy seems the most promising for the development of new powerful therapeutic strategies for cancer treatment.

1.2.4 Circulating microRNA

Biopsy and imaging examination represent the conventional gold standard for cancer detection, together with the analysis of general tumor markers, including carcinoembryonic antigen (CEA) and CA199. However, these available tools are rather invasive and low sensitive, whereby the identification of new non-invasive and high sensitive biomarkers represents the most important challenge to achieve early diagnosis and substantial improvement of patients' outcome and mortality rate (Wang H, 2018). In recent years, the discovery of miRNAs in biological fluids has generated great interest for their potential use as biomarkers, given their modulation associated with specific biological/pathological conditions. Circulating biomarkers play a significant role in clinical application for diagnosis, treatment response monitoring, or tumor recurrence prediction (Kosaka N, 2010). Recent evidences have revealed that miRNAs are routinely secreted by cancer cells into biological fluids, such as serum and plasma, saliva, urine and breast milk (Chen X, 2008) (Lodes MJ, 2009). They can impact not only in the tissue of origin but also in the regulation

of gene expression of distant target cells (Ferracin M, 2015). Circulating miRNAs are released into bloodstream in many forms and the reasons for their high stability remain largely unknown, although many hypotheses have been proposed (Cortez MA, 2011):
i) circulating miRNAs may have unique modifications, such as methylation, adenylation, and uridylation, that could increase their stability protecting them against RNAses (Katoh T, 2009); ii) they are protected by encapsulation in cell-derived microvesicles (Valadi H, 2007) or iii) through specific RNA-binding proteins (Arroyo JD, 2011) (Kosaka N, 2010).
Despite their origin and release have not been fully elucidated, three different mechanisms are suggested (Cortez MA, 2011): i) passive release from apoptotic or necrotic cells, as well as from damaged tissues, or from cells with a short half-life, such as platelets; ii) active secretion through cell-derived microvesicles, including exosomes and shedding vesicles. The exosomes are formed through the internal budding of the first endosomal membranes, giving rise to intracellular multivesicular bodies which blend with the plasma membrane and are then released into the extracellular environment. Shedding vesicles are larger vesicles generated by a plasma membrane extroflexion (Cortez MA, 2011). iii) The last hypothesis involves the active cellular secretion of both free and RNA binding protein-complexed miRNAs. The latter are represented by nucleophosmin (the nucleolar phosphoprotein NMP1) (Cortez MA, 2011), high/low density lipoproteins (Vickers KC, 2011) and the Argonaute protein family (AGO) (Valadi H, 2007) (Arroyo JD, 2011) (Wang K, 2010) (**Figure 9**).

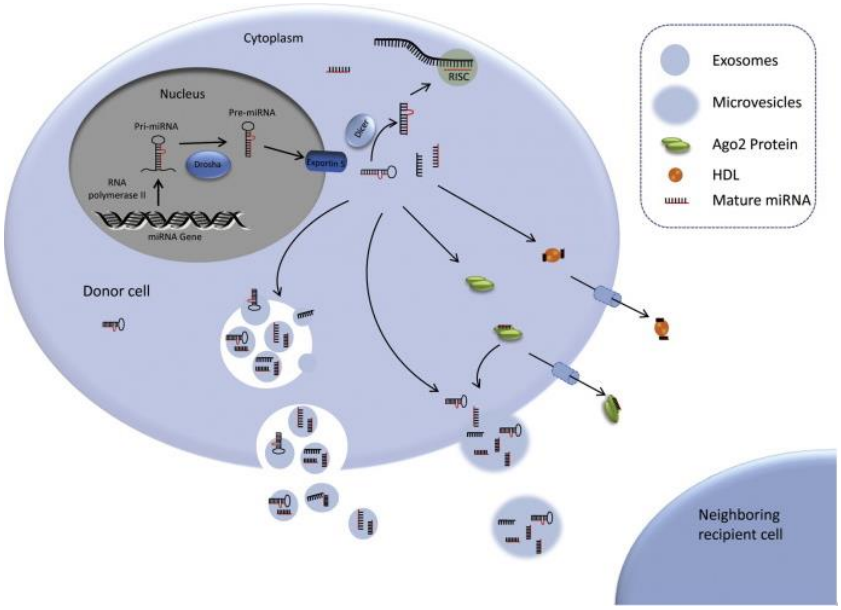


Figure 9 Release of miRNAs in the Extracellular Environment. Mature miRNAs can be selectively incorporated into the exosomes or coupled with Ago2 protein and released into extracellular fluids. Alternatively, they can be enwrapped with microvesicles or attached to HDL and later released to extracellular environment. (HasanSohel, 2016).

Taking into account what has recently been described in the scientific literature, circulating miRNAs have many of the essential characteristics of a good biomarker: they are stable in circulation, resistant to RNase digestion, extreme pH, high temperatures, prolonged storage and multiple freeze-thaw cycles (Chim SS, 2008) (Ge Q, 2014). Nevertheless, the clinical efficacy of miRNAs as circulating biomarkers can be affected by a number of variables such as sample collection and processing, RNA extraction efficiency, as well as other technical aspects involved in the success of qRT-PCR and data analysis; however, the correct performance of all operations ranging from blood collection to RT-PCR data analysis, can increase the sensitivity allowing to obtain reliable results (Kroh EM, 2010).

In the last years, thanks to the use of increasingly sophisticated techniques, such as Microarrays or low-density arrays, it has been detected a number of miRNAs differentially expressed in body fluids of cancer patients compared to healthy volunteers, demonstrating the possibility to identify a miRNA signature representative of a specific pathological condition.

The profiling of circulating miRNAs could be useful as minimally invasive diagnostic and prognostic biomarker for various cancers including lung, prostate, breast, colon, gastric, cervical, head and neck cancers (Wang H, 2018) (Hou B, 2015). For example, plasma miR-145, miR-20a, miR-21, and miR-223 were significantly up-regulated in the early stage of non-small cell lung cancer (NSCLC) (Zhang H, 2017). Moreover, Zeng *et al.* demonstrated that serum miR-17, miR-20a, miR-29c, and miR-223 expression levels were significantly different between nasopharyngeal carcinoma patients and non-cancerous volunteers, and these differences were effective to distinguish the malignancy with high sensitivity and specificity (Zeng X, 2012). In another report, serum miR-182 and miR-331-3p were significantly increased in hepatocellular carcinoma patients, and the diagnostic and prognostic abilities of these miRNAs were high in receiver-operating characteristic (ROC) curve analysis (Chen L, 2015).

1.3 Molecular Biomarkers for Laryngeal Cancer

Cancer patients with similar clinical and anatomic-pathological parameters can often have different prognosis (Golusinski W, 1999). Many studies are focused on proteins, genes or chromosomes, whose aberrations in neoplastic tissues could correlate with the biological behaviour of disease. Particularly, the molecules of interest act as regulators of normal and cancer cells development and can be divided in three categories: pro-oncogenes, growth factors and onco-suppressive genes. The understanding of the downstream molecular changes of such aberrations could help in clarifying the mechanisms underlying cancer progression and, above all, it could direct the identification of specific biological targets for the development of new treatment strategies.

The main pathological pathways implicated in LSCC tumorigenesis include the dysregulation of cellular survival and proliferation (Tp53 and EGFR), cell-cycle control (CDKN1A) and cellular differentiation (NOTCH1) (Zhou G, 2016) mediators. Cyclin D1 is one of the most studied proto-oncogenes that form, together with cyclin-dependent kinases cdk4 and cdk6, a complex able to phosphorylate the RB protein, thus implying cell cycle progression from G1 to S phase. Cyclin D1 overexpression seems to correlate with a worse prognosis of laryngeal carcinoma (Fracchiolla NS, 1997). The onco-suppressor p53 acts at various levels by regulating gene transcription, inhibiting cell cycle and promoting apoptosis in damaged cells after exposure to radiation or chemotherapy. p53 overexpression was

demonstrated to positively correlate with advanced LSCC stage (Chrysovergis A, 2019). Moreover TP53 gene mutations have been assumed to affect the response to radiotherapy and to drive both cell differentiation and neck lymph-node metastasis (Osman I, 2002) (Zhang X, 2002). Together with p53, Bcl-2 plays a central role in the regulation of apoptosis. It belongs to Bcl-2 family members which act either as anti-apoptotic (Bcl-2 and Bcl-xL) or as pro-apoptotic (Bax and Bak) mediators. It has been proven that p53/Bcl-2 co-expression was significantly associated with poor differentiation, tumor extension, lymph-node metastases, and advanced clinical stage of LSCC (Pruneri G, 1998). More recently, research interest has shifted to the p27 protein. It inhibits G1 to S phase cell cycle progression by binding to the cyclin-dependent cyclin kinase complex. High p27 expression was preliminarily correlated with a better prognosis in the case of LSCCs (Calgaro N, 2007). Instead, reduced p27 expression in LSCC was correlated with aggressive behaviour, advanced clinical stage, and metastatic disease (Fan GK, 1999) (Tamura N, 2001).

Endothelial Growth Factor Receptor (EGFR) and Vascular Epithelial Growth Factor (VEGF) are included in the big family of growth factors. EGFR is an approximately 170 KD trans-membrane glycoprotein belonging to the tyrosine kinase receptor class. EGFR signal is essential for both development and homeostasis of normal tissues and the activity of some members of this family, but it plays a key role also in the development and growth of cancer cells. EGFR levels seem, in fact, to be directly correlated with the severity of the head and neck carcinomas, since EGFR activation can stimulate proliferation, angiogenesis, protection from apoptosis, loss of differentiation, migration and invasion, all essential elements in malignant neoplasms development (Cheng J, 2004). Furthermore, the evaluation of EGFR, Cyclin D1 and KRAS in LSCC has demonstrated that their overexpression is closely associated with clinical stage, metastasis, recurrence after treatment and survival, highlighting their synergistic role in the onset and development of LSCC, as well as in worse prognosis (Lin X, 2019). VEGF protein is secreted by hypoxic cells and acts through a paracrine mechanism by stimulating endothelial cell migration and proliferation, thereby inducing angiogenesis also in neoplastic cells (Riedel F, 2000).

NOTCH signaling pathway plays a central role in embryonic development and adult life, as well as in tumor biology regulation. Actually, NOTCH1 activity is contextual, playing a bimodal role as tumor suppressor or oncogene (Fukusumi T, 2018). Some works support its role in LSCC malignant progression and lymphatic metastases (Li D, 2014); Dai *et al*, demonstrated also its contribution in migration and invasion of LSCC cell lines. Nevertheless, further studies are warranted to determine the molecular mechanisms

underlying NOTCH1 role in LSCC invasion and metastasis (Dai MY, 2015). In has been showed that also NOTCH2 signaling contributes to cell growth, survival and metastasis in LSCC (Zou Y, 2016). Mutations in two of the most important intracellular regulators of cell growth, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) and FGFR3 (Fibroblast growth factor receptor), have recently been detected in LSCC with progressing dysplasia, while mutations in JAK3 (Janus Kinase 3), MET and FBXW7 (F-Box and WD Repeat Domain Containing 7) were found only in non-progressing dysplasia patients (Manterola L, 2018). PTEN is a tumor suppressor gene, member of the PI3K/AKT/PTEN/mTOR signaling pathway, which regulates many cellular functions including cell proliferation, protein synthesis and survival. It has been demonstrated a specific correlation between decreased PTEN expression and LSCC glottic localization, while a lower association has been showed in comparison with the supraglottic one. PTEN down-regulation was also associated with tumor aggressiveness, showing a correlation with shorter survival and advanced tumor stage (T2-T3) (Mastronikolis NS, 2017). PARK7, (Parkinson protein 7) known as DJ1, acts as a positive regulator of androgen receptor-dependent transcription and its up-regulation is related to lymph nodal status, clinical stage, and patients' outcome (Zhu XL, 2012). In particular, DJ-1 overexpression is negatively correlated with PTEN expression in LSCC tumor tissues; DJ-1-induced PTEN downregulation affects proliferation and invasion of LSCC (Kim RH, 2005).

Epithelial Mesenchymal Transition (EMT) is a process linked to PI3K/AKT signal pathway. TrkB (tropomyosin-related kinase B receptor) is frequently overexpressed in highly metastatic laryngeal cancer cell lines and in clinical laryngeal cancer samples, where it acts as a key regulator of the c-Src-mediated activation of PI3K/AKT signal pathway and promotes EMT (Jiang L, 2017). Even though the underlying mechanism remains unclear, the altered expression and the occurrence of genetic mutations affecting TrkB have been proved to play an essential role for tumor progression, as observed by the increase of invasion, metastasis, angiogenesis, and resistance against therapeutic treatments.

In the last twenty years, EMT has been increasingly studied, highlighting the main difference between migration of normal keratinocytes and transformed epithelial cells. This process regards exclusively cancer cells and it is characterised by adherents' junctions breaking and epithelial markers' (*i.e.* cytokeratins and E-cadherin) reduced levels, accompanied by mesenchymal markers' (*i.e.* fibronectin, N-cadherin, and Vimentin) upregulation (Ribatti D, 2017).

Cadherin switching, typical of malignant tumors, including HNSCC, is often correlated with the occurrence of lymph node metastasis (Jung AR, 2020). In LSCC, it was identified a significant association between E-cadherin expression and tumor site, differentiation, stage and lymph nodal involvement. In detail, reduced E-cadherin expression correlated with poorly differentiated supraglottic tumors at advanced stages (Ahmed RA, 2014). A similar observation was reported for E-cadherin and β-catenin in LSCC patients: decreased expression levels of both proteins leaded to a greater local aggression and cervical metastases occurrence (Nardi CE, 2018). Besides the evaluation of the E-cadherin/N-cadherin, E-cadherin/β-catenin co-expression and their role in oncogenesis, development and metastasis, it has been assessed also the simultaneous expression of E-cadherin and ZEB2 in LSCC. The latter is a transcriptional repressor that induces EMT by negatively regulating E-cadherin expression, thus promoting tumor invasiveness. Some evidence report ZEB2 as a critical factor in predicting LSCC prognosis (Zhu GJ, 2018). All these molecular changes allow cancer cells to gain an invasive fibroblastoid phenotype, but EMT can also concur to the modulation of other aspects of cancer progression, for example the decreased susceptibility of mesenchymal cells to immune clearance, as a result of reduced physical connection between immune and target cells, which acquire a characteristic phenotype known as mesenchymal immune evasion (MIE) (Terry S, 2017). The main actor in this condition is the transforming growth factor-beta (TGF-β), which can change the tumor microenvironment (TME) in the direction of a tumor-supportive environment through the enhancement of immunosuppression by regulatory T cells (T-regs) activation (Terry S, 2017). Nowadays, it is evident that cell proliferation could cause the onset of cancer if it is sustained and if it occurs in an environment rich of pro-inflammatory cells and mediators, growth factors, activated stroma, and DNA-damage-promoting agents, including cytokines, chemokines, cyclooxygenase-2, prostaglandins, fibroblast growth factor (FGF), MMPs and the already mentioned VEGF (Coussens LM, 2002). Among the pro-inflammatory cytokines, IL-6, together with IL-1 and TNF-α, is one of the most relevant promoters of acute inflammatory response, also reinforcing chronic phase inflammation, thus providing the favourable environment for tumor growth. IL-6 is secreted by B and T lymphocytes, macrophages, fibroblasts, keratinocytes, and also by cancer cells, controlling both proliferation and apoptosis processes (Mojtahedi Z, 2011), in fact its levels have been significantly correlated with tumor invasion, severity, spreading and chemo-resistance (Duffy SA, 2008). Il-6 activates the JAK/STAT axis, which in turn enhances metastasis via

EMT induction, increases motility via focal adhesion kinase (FAK) and improves tumor invasion through the activation of VEGF and rho (Yadav A, 2011).

So far, many reports showed the aberrant regulation of various miRNAs in LSCC tissues and/or biological fluids, in association with tumor onset or development, due to their interaction with the main crucial factors involved in survival, death, differentiation and metastasis pathways (Ricciardiello F, 2017) (Cao P, 2013). MiR-141, for example, has been found downregulated in LSCC tissues of patients with nodal metastasis and high TNM stage. The molecular mechanism was characterized both *in vitro* and *in vivo*, showing that miR-141 inhibits EMT, lymph node metastasis (LNM) occurrence and TGF-β pathway, through direct targeting of Homeobox C6 (HOXC6), a well-known EMT regulator (Chen L, 2020). Likewise, miR-138 suppresses LSCC cells' ability to spread into adjacent tissues, regulating the invasion factor ZEB2, whose expression decreased in LSCC tissues with distal metastasis (Gao S, 2015). Low miR-145 levels also were related with poor prognosis, promoting cell proliferation and invasion, as well as inhibiting apoptosis through the direct targeting of MYO5A (Zhao X, 2018). Many other miRNAs in a similar way are involved in LSCC aggressiveness: miR-203 via SAP1 (Tian L, 2014), miR-204-5p by targeting Forkhead box C1 (FOXC1) (Gao W, 2017), miR-143-3p through the involvement of k-Ras/Raf/MEK/ERK signaling pathway (Zhang F, 2019). Down-modulated miR-101 exerts its tumorigenic effect by Cyclin-dependent kinase 8 (CDK8) targeting (Li M, 2015), while miR-195 inhibits cell proliferation, colony formation, migration and invasion, promoting cell cycle arrest and apoptosis by direct targeting of DCUN1D1, known as a squamous cell carcinoma-related oncogene (Shuang Y, 2017).

Recent evidence has also focused on the upregulation of different microRNAs in LSCC progression. miR-16 acts as an oncomiR in LSCC cells via direct targeting of Zyxin, which regulates cell migration and adhesion (Wu H, 2011). Moreover, Min Liu *et al*. demonstrated that miR-21 up-regulation in LSCC tissues enhances cell growth by suppressing BTG2, which is known as pan-cell cycle regulator and tumor-suppressor (Liu M, 2009). In addition, Yuan Tian *et al*. identified miR-27a as an oncogenic miRNA in LSCC, revealing its capability to increase cell viability and colony formation, as well as to inhibit apoptosis through the down-regulation of the tumor-suppressor PLK2 (Tian Y, 2014). Similarly, miR-155 increased LSCC cells' proliferation and invasion via targeting both SOCS1 and STAT3 (Zhao XD, 2013), meanwhile miR-744-3p enhanced the pro-metastatic ability of laryngeal cancer cells through Programmed cell death 4 (PDCD4) and PTEN targeting, leading to Matrix metallopeptidase 9 (MMP-9) activation, thus enabling cancer

cell migration (Li JZ, 2016). Also miR-223 has been showed as positive regulator of LSCC cell growth, as demonstrated by the evidence of pERK2, pAKT and AKT induction (Bozec A, 2017).

Our research group has performed a high-throughput microarray analysis for miRNA expression profiling in laryngeal cancer tissues, defining a predictive miRNA signature for nodal metastases occurrence. In detail, we identified seven up-regulated (miR-618, miR-542-5p, let7b, miR-135a, miR-20b, miR-324-3p and miR-886-5p) and four down-regulated (miR486-3p, miR-328, miR-376a and miR-493) miRNAs in N^+ group compared with N^- (Ricciardiello F, 2017). A further characterization, by ROC curve analysis, displayed the strong potential of miR-449a as predictive marker of nodal involvement. We also proposed a possible mechanism of action via Notch1 and 2 direct targeting and functional *in vitro* assays demonstrated miR-449a-mediated inhibition of LSCC cell proliferation, migration, and invasion (Kawasaki H, 2020).

1.4 Physiological and Pathological Role of miR-223

MiR-223 is a short RNA molecule located within the q12 locus of the X chromosome governed by an independent promoter, not related to other gene products. miR-223 is highly conserved, suggesting its potential role in essential physiological events. It is preferentially expressed by hematopoietic cells, especially $CD34^-$ bone marrow cells (Fazi F, 2005), where it is mainly involved in myeloid linage development (Sun W, 2010) (Johnnidis JB, 2008), promoting granulocytic differentiation (Johnnidis JB, 2008) (Fazi F, 2007) and suppressing erythrocytic one (Yuan JY, 2009). *MiR-223*$^{-/y}$ mice have been studied in several murine disease models to investigate the impact of miR-223 in innate immunity, reporting no differences between male and female miR-223 deficient mice, but showing a profound peripheral blood neutrophilia in hemizygous *miR-223*$^{-/y}$ mice (Johnnidis JB, 2008). The crucial role of miR-223 in haematopoiesis has been extensively analysed in the past few years, highlighting its involvement as fine regulator of granulocytic differentiation, maturation and function (Felli N, 2009). In detail, human granulocytic differentiation is controlled by miR-223 and two transcriptional factors, NFIA and C/EBPα. NFIA maintains miR-223 at low levels, whereas C/EBPα upregulates miR-223 expression. The competition by C/EBPα and the granulocytic differentiation are favoured by a negative-feedback loop in which miR-223 represses NFIA translation (Fazi F, 2005). Contrariwise miR-223 down-

regulation during erythropoiesis is required for erythrocyte proliferation and differentiation at progenitor and precursor level (Felli N, 2009).

Pathologically, miR-223 can alter cancer cell phenotypes not only in haematological malignancies but also in solid ones. Many evidences have revealed pleiotropic effects of miR-223 in different types of cancer. It is commonly repressed in acute myeloid leukemia (AML) (Fazi F, 2007), cervical cancer (Tang Y, 2015) and non-small cell lung cancer (NSCLC) (Zhao FY, 2016), acting as a tumor suppressor, whereas higher expression levels of miRNA-223 are associated with some cancer types, such as T-cell acute lymphoblastic leukemia (T-ALL) (Mavrakis KJ, 2011), gastric cancer (Li J, 2012) and prostate cancer (Wei Y, 2014), were it functions as an oncomiR. The role of miR-223 in carcinogenesis has still not been fully characterized and understood, but abnormalities in its expression pattern are correlated with a broad variety of cancer-related cellular processes, including cell proliferation, apoptosis, differentiation, metastasis and invasion (**Table 1**). So, with respect to its role in carcinogenesis, miR-223 could serve as a promising biomarker for diagnosis and differential diagnosis, tumor staging and prognosis evaluation. For example, miR-223 overexpression in gastric cancer is associated with lymph node metastasis or metastatic disease (M1) at an advanced pathological stage (Li J, 2012). Laios *et al* demonstrated that miR-223 was heavily expressed in ovarian cancer tissues, particularly in recurrent tumor samples (Laios A, 2008).

Cancer Type	*Target genes*	*Related function*
Acute myelocytic leukemia	E2F1	Cell circle
Breast cancer	Caprin-1, EGF, Mef2c	Proliferation, invasion, metastasis
	STMN-1	Chemotherapy-resistant
Cervical cancer	FOXO1, IGF1R, RASA1	Proliferation
Childhood acute lymphoblastic leukemia	E2F1, CEBP-α, E2A	Cell cycle
Colorectal cancer	FOXO1, RASA1	Proliferation, growth
	STMN-1	Chemotherapy-resistant
Chronic lymphocytic leukemia	HSP90B1	Apoptosis
Endometrial cancer	IGF1R	Proliferation, cell circle
Esophageal cancer	ARTN, FXBW7, PARP1, PDCD4, STK40	Proliferation, cell cycle, growth, viability, apoptosis, invasion, metastasis
Gastric cancer	BCL2, DNMT3B, FBXW7/hCdc4, EPB41L3, IGF1R, STMN1	Proliferation, growth, apoptosis, invasion, metastasis
	FBXW7	Chemotherapy-resistant
	Sp1	EMT
Glioblastoma	ATM	Radio-sensitivity
Hepatocellular cancer	FOXO1, STMN1	Proliferation, cell circle, apoptosis
Juvenile myelomonocytic leukemia	LMO2, CEBP-β	Proliferation, metabolism, development
Lung cancer	CDK2, EPB41L3, IGF1R	Proliferation, invasion
	FBW7, IGF1R	Chemotherapy-resistant
Mycosis fungoides	Ect2, MEF2C, TOX	Proliferation, clonogenic potential
Nasopharyngeal cancer	MAFB	Proliferation and migration
T-ALL	FBXW7, IGF1R	Proliferation, apoptosis
Oral squamous cell cancer	IGF1R, RHOB, STMN1	Proliferation, apoptosis
Osteosarcoma	Ect2, HSP90B1	Proliferation, cell circle, growth, apoptosis
Penile cancer	SLC8A1	Proliferation, apoptosis
Pleural mesothelioma	STMN1	Motility and proliferation
Prostate cancer	ITGA3, ITGB1, SEPT6	Proliferation, invasion, migration
Vulvar cancer	TP63	Proliferation, invasion

Table 1 miR-223 Targets and their Related Functions in Cancer (Gao Y, 2017)

In addition, due to distinct functions in tumor initiation, progression and metastasis, miR-223 can also be considered as a novel drug target or therapeutic tool for human cancer treatments (**Figure 10**). miR-223 based treatments have two possible approaches: miR-223 replacement or reduction, both may be obtained using various miRNA delivery systems,

such as viral vectors, nanoparticle-based delivery, chemically modified mimics, sponges, and antagomiRs. Wei *et al* demonstrated that knocking down miR-223 in prostate cancer cells resulted in cell apoptosis, G0/G1 arrest and decreased invasive ability (Wei Y, 2014). Furthermore, miR-223 inhibitor combined with other anti-cancer drugs could exert synergistic therapeutic effects (Ma J, 2016).

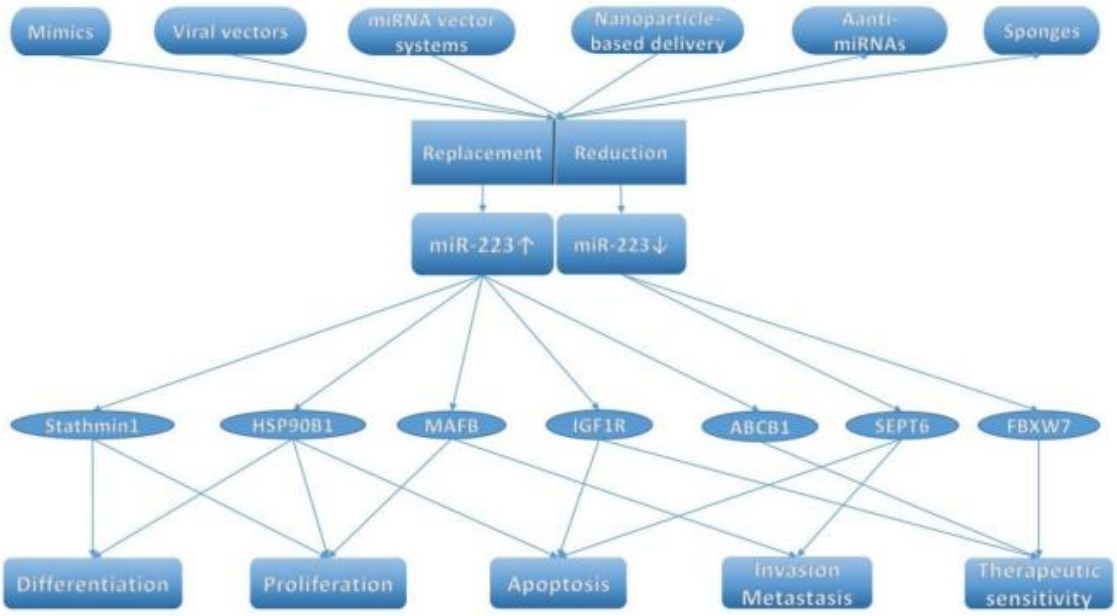


Figure 10 miR-223-based Treatments. A promising approach to treat different types of cancers with miR-223 replacement or reduction (Gao Y, 2017).

II AIM OF THE WORK

LSCC is an important oncologic entity, whose prognosis mainly depends on prevention and appropriate diagnosis, especially for high-risk population. Despite major efforts to maximize the possibility of complete resolution for initial stages, late-stage disease has still a worse outcome because of the absence of useful prognostic factors. So, early diagnosis is still a challenge, and the identification of non-invasive molecular markers could represent a powerful strategy to counteract the high morbidity and mortality of this neoplasm. Recently, microRNAs have been evaluated for their potential role as biomarkers or therapeutic targets due to their frequent deregulation in tumor tissues and body fluids including blood, urine, saliva. They play critical roles in tumor suppression or carcinogenesis by regulating gene expression at post-transcriptional level. Therefore, gene therapies based on miRNA inhibition or replacement could represent a new useful strategy for the treatment of neoplastic diseases. The basic premise of the present work was to investigate miRNA expression profile in LSCC sera for the identification of new reliable and specific non-invasive biomarkers for LSCC diagnosis and prognosis, as well as for the possible identification of therapeutic candidates. Particularly, we collected sera samples from both LSCC patients and healthy donors, thanks to the collaboration of 4 different hospitals in Campania. All these samples were integrated with clinical data on tumor stage and grade. The comparison between LSCC patients and healthy group or, within LSCC group, between the nodal metastatic and non-metastatic ones allowed us to define and characterize a possible serum miRNA signature for LSCC diagnosis. Besides the diagnostic role, deregulated miRNAs were also investigated for their putative targets and the inherent pathways. Among the up-regulated serum miRNAs miR-93, miR-223 and miR-532, we focused on the biological and molecular role of miR-223, whose increased expression was also found at tissue level in LSCC patients, as reported in our previous work. In detail, based on the availability of LSCC tissue miR-223 levels, we correlated miRNA expression with tumor extension, highlighting a significant up-regulation in patients with advanced cancer (T3-T4), compared to early stage patients (T1-T2), thus suggesting a possible correlation between miR-223 levels and tumor volume. Then we dissected *in vitro* the role of miR-223 using a preclinical LSCC model and analyzing the molecular effects of its deregulation on viability, clonogenicity and migration, identifying a global oncogenic function.

Collectively, our findings suggest that serum miR-93, miR-223 and miR-532 could be biomarker candidates for LSCC diagnosis, and miR-223, working as oncomiR, could represent a possible target for miRNA-based therapy.

III RESULTS

3.1 Definition of Serum miRNA Signature and its Diagnostic Value for LSCC

To determine new reliable non-invasive biomarker candidates for the definition of LSCC diagnosis and prognosis, as well as for the identification of new therapeutic targets, we investigated serum miRNA expression profile perturbation in LSCC patients. A high-throughput PCR array analysis was performed on miRNAs extracted from a cohort of 45 LSCC patients, including 22 with lymph nodal metastases (N^+) and 23 without (N^-), compared with 23 healthy donors. Using TaqMan Array Human MicroRNA A Cards v2.0 (Applied Biosystems) we evaluated the simultaneous expression of 377 mature miRNAs; the differential expression patterns of the 81 detected miRNAs was represented with a hierarchical clustering heatmap (**Figure 11**).

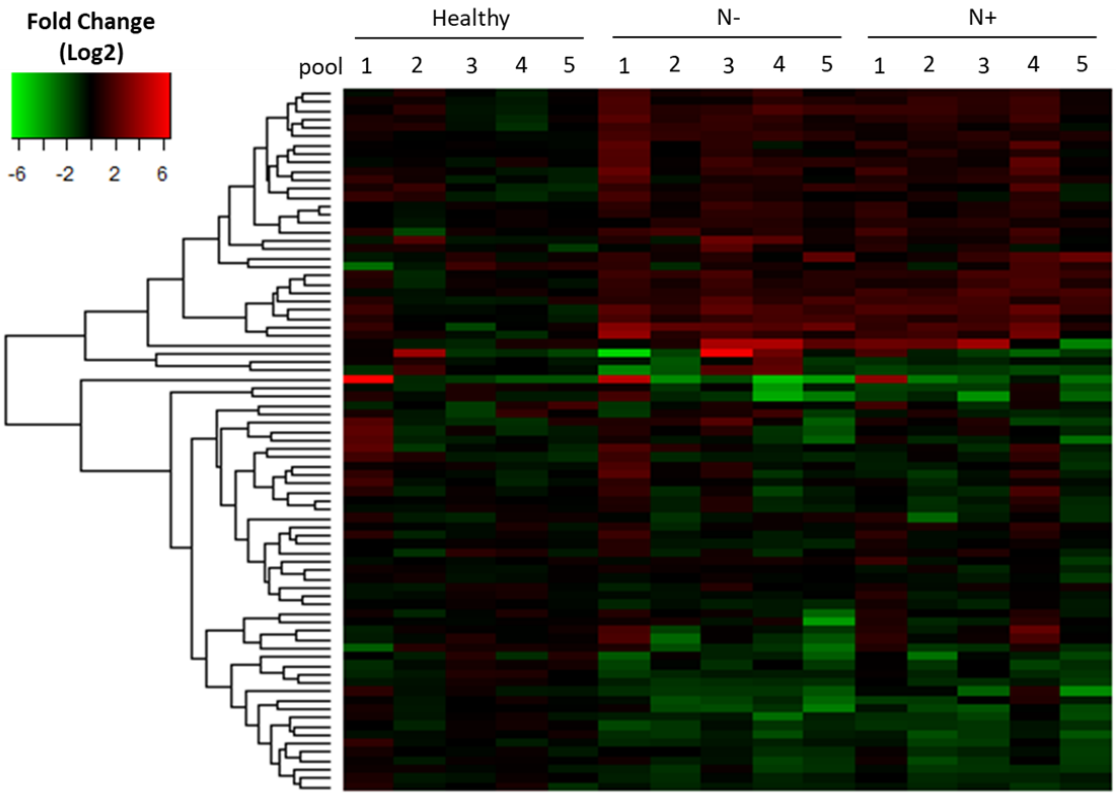


Figure 11 Serum miRNAs Global Expression Pattern among N^+ or N^- LSCC Tissue Samples Compared to Healthy Donor Groups. The hierarchical clustering heatmap summarizes the 81 detectable serum miRNA expression levels, normalized by miR-222 as a reference endogenous control.

The statistical analysis of the obtained data set allowed us to evaluate the significant expression difference among the groups. In detail, three inclusion criteria have been set: (1) Mean Ct < 32.0, (2) Mean Fold change (Log2) < -1.0 or >1.0, (3) *p*-value < 0.05 in order to select reliable miRNAs dysregulated from the 81 candidates. Applying the following criteria, 11 up- and 5 down-regulated miRNAs emerged in the comparison between cancer and healthy groups, while no significant differences were highlighted between N^+ and N^- groups (**Table 2**).

miRNA	Regulation	Fold change (Log2)	S.D.	95% CI	p-value	q-value
miR-532	Up	2.1	0.7	1.7 - 2.5	0.001	0.014
miR-93	Up	1.8	0.4	1.5 - 2.1	0.000	0.014
miR-451	Up	1.5	1.1	0.8 - 2.1	0.021	0.187
miR-140	Up	1.4	0.4	1.1 - 1.6	0.001	0.014
miR-223	Up	1.3	0.4	1.1 - 1.6	0.000	0.014
miR-16	Up	1.3	0.4	1.0 - 1.6	0.001	0.014
miR-20b	Up	1.3	0.5	1.0 - 1.6	0.001	0.090
miR-29a	Up	1.3	0.5	1.0 - 1.5	0.001	0.090
miR-132	Up	1.1	0.9	0.6 - 1.7	0.030	0.253
miR-25	Up	1.0	0.6	0.7 - 1.4	0.011	0.187
miR-20a	Up	1.0	0.8	0.5 - 1.5	0.039	0.253
miR-95	Down	-1.0	1.0	-1.6 - -0.4	0.050	0.253
miR-150	Down	-1.1	0.4	-1.4 - -0.9	0.001	0.014
miR-891a	Down	-1.1	0.8	-1.6 - -0.7	0.013	0.187
miR-331	Down	-1.2	0.6	-1.6 - -0.9	0.001	0.090
miR-374	Down	-1.4	1.0	-2.0 - -0.8	0.013	0.187

Table 2 Differential Expression of Serum miRNAs in LSCC Patients

Based on fold change and its statistical significance, we focused on three up-regulated miRNAs - miR-93, miR-223, and miR-532 - as possible diagnostic marker candidates to be validated in a small population of 10 LSCC patients and 8 healthy donors (**Figure 12**). Once confirmed their significant up-regulation (miR-93 $p < 0.01$, miR-223 $p < 0.001$, miR-532 $p < 0.001$), consistently with the microarray result, we carried on a further validation on an increasingly large study population. To date, totally 75 LSCC patients (43 N^- and 32 N^+) and 29 healthy volunteers were enrolled for the validation test.

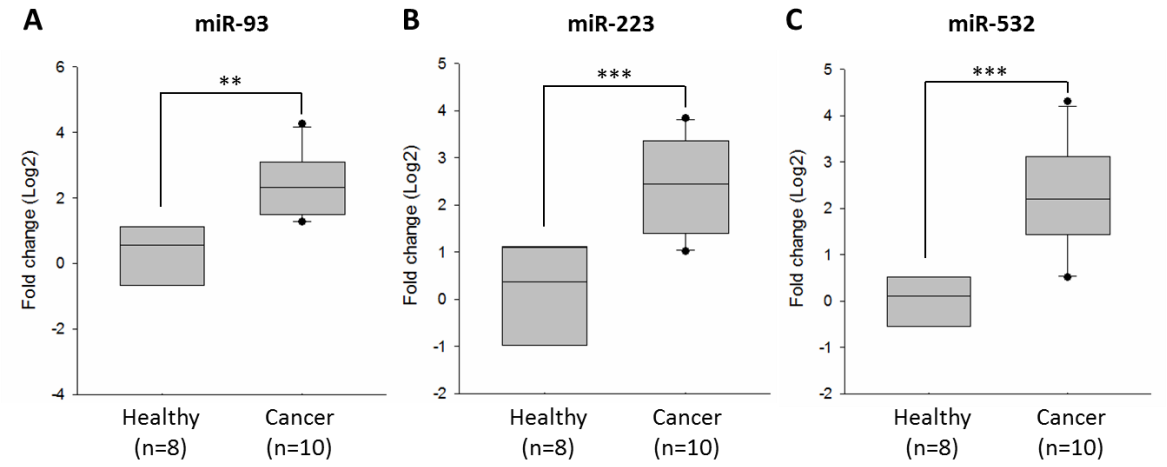


Figure 12 The Expression Levels of Validation Candidates in a Small Cohort. The expression of (**A**) miR-93, (**B**) miR-223, (**C**) miR-532, in LSCC group (n=10) and healthy one (n=8) were measured by qRT-PCR. Exogenous cel-miR-39 was used as a normalizer. The *p*-value was calculated by t-test, ***p* <0.01, ****p* <0.001.

In **Table 3** we summarized the clinic-pathological features of both discovery and validation cohorts.

Clinical features	Discovery cohort	Validation cohort
Number of patients	45	75
Mean age (years ± S.D.)	60.1 ± 9.8	63.2 ± 10.6
≤60	23 (51.1)	33 (44.0)
>60	22 (48.9)	42 (56.0)
Gender		
Male	42 (93.3)	65 (86.7)
Female	3 (6.7)	10 (13.3)
T classification		
T1-T2	9 (20)	22 (29.3)
T3-T4	36 (80)	53 (70.7)
Lymph node metastasis		
Negative	23 (51.1)	43 (57.3)
Positive	22 (48.9)	32 (42.7)

Table 3 Clinical Data of LSCC Patients

By RT-qPCR analysis we confirmed the significant up-regulation of miR-93 (Median fold change = 4.2, *p*-value = 1.3×10^{-5}), miR-223 (Median fold change = 3.4, *p*-value = 6.1×10^{-5}), and miR-532 (Median fold change = 3.9, *p*-value = 9.2×10^{-7}) in cancer group compared to the healthy one (**Figure 13**).

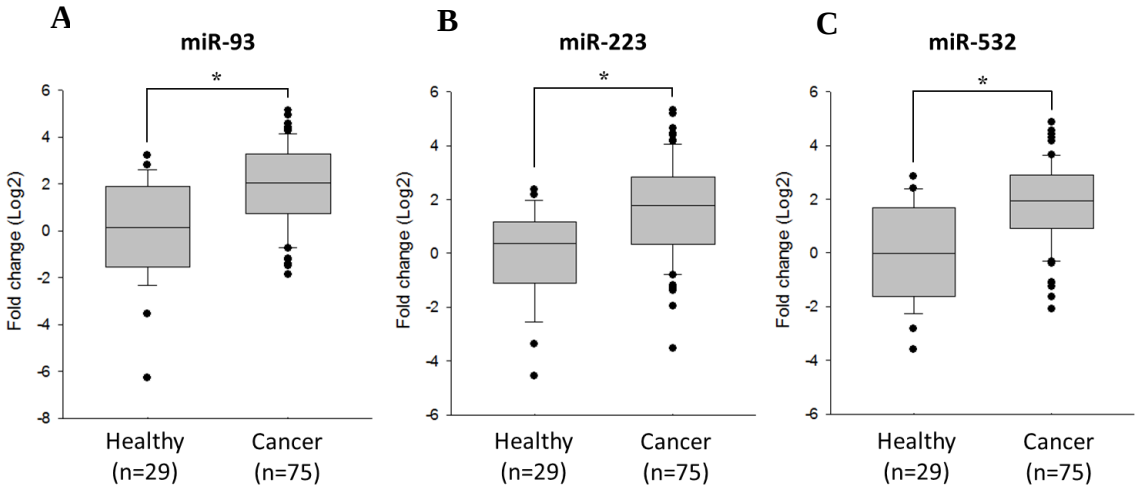


Figure 13 Serum miR-93, miR-223, and miR-532 Expression Levels in LSCC Patients. The expression levels of serum miR-93 (**A**), miR-223 (**B**), and miR-532 (**C**) in LSCC patients (n = 75) compared with healthy donors (n = 29), validated by qRT-PCR. Exogenous cel-miR-39 was used as normalizer. The *p*-value was calculated by t-test *p <0.001.

To define the potential diagnostic value of serum miR-93, miR-223 and miR-532 levels, the receiver operating characteristic (ROC) curve was plotted. This technique served to identify a diagnostic threshold value for miRNA candidates. The area under the ROC curve (AUC) was 0.75 for miR-93, 0.74 for miR-223 and 0.78 for miR-532. The diagnostic sensitivity, specificity and accuracy values calculated for each one [miR-93 (95% CI = 0.65 - 0.85, Sensitivity = 0.99, Specificity = 0.28, Accuracy =0.79), miR-223 (95% CI = 0.65 - 0.84, Sensitivity = 0.97, Specificity = 0.21, Accuracy =0.76) and miR-532 (95% CI = 0.68 - 0.87, Sensitivity = 0.99, Specificity = 0.24, Accuracy =0.78)], showed their moderate diagnostic ability (**Figure 14**).

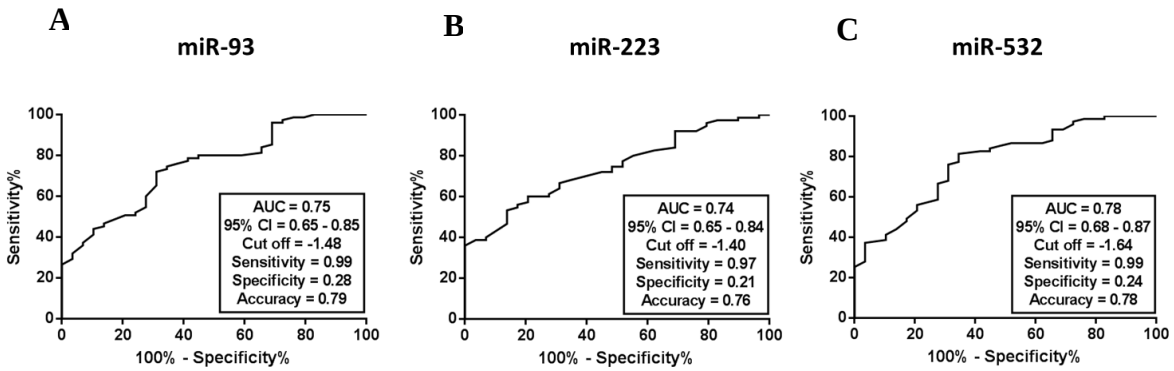


Figure 14 ROC Curve Analysis for Serum miR-93, miR-223, and miR-532. Receiver operating characteristic (ROC) curve analyses demonstrated LSCC diagnostic ability of miR-93 (**A**), miR-223 (**B**) and miR-532(**C**). AUC, sensitivity, specificity, and accuracy was examined comparing fold change (Log2) between LSCC (n=75) and healthy cohort (n=29).

Our goal was not only to identify potential diagnostic miRNAs, but also to be able to exploit their impact on prognosis. For this reason and based on our findings, we analysed a possible statistical relation between selected miRNAs' expression levels and LSCC patients' clinic-pathologic features by calculating the correlation coefficient (*r*) and *p*-value. Interestingly, results showed a weak negative correlation between both miR-93 and miR-532 expression levels and tumor stage (T) [r = -0.19 (p-value < 0.039) and r = -0.28 (p-value < 0.014), r espectively], suggesting their potential diagnostic value in detecting tumor at early stages; no other significant correlations with age, gender or lymph node metastasis involvement were detected (**Table 4**).

		miR-93			miR-223			miR-532		
Clinical Feature	Patients (n)	Mean Log2 FC	r	p value	Mean Log2 FC	r	p value	Mean Log2 FC	r	p value
AGE			-0.10	0.51		-0.23	0.12		-0.02	0.92
≤60	33	2.0			2.0			2.1		
>60	42	1.8			0.68			1.7		
GENDER			-0.069	0.45		-0.061	0.65		-0.34	0.77
Male	65	1.9			1.6			1.9		
Female	10	1.5			1.4			1.7		
TUMOR STAGE			-0.19	0.039		-0.15	0.22		-0.28	0.014
T1-2	22	2.4			2.0			2.4		
T3-4	53	1.7			1.4			1.6		
LNM			-0.34	0.084		0.020	0.79		-0.088	0.45
N0	43	1.9			1.6			2.4		
N1-4	32	1.8			1.7			1.6		

Table 4 Clinic-pathological Correlation of Serum miR-93, miR-223 and miR-532 for LSCC

At this point we decided to analyse *in vitro* the molecular effects of miR-223 deregulation using two LSCC cell models. The choice to deepen the role of this miRNA derives from the observation that, among the serum miRNAs identified, it was the only one which resulted significantly up-regulated ($p<0.001$) at the tissue level too, as we recently reported (Ricciardiello F, 2017). Starting from this concept, we performed a statistical analysis on our data set finding a huge miR-223 up-regulation in patients with advanced cancer stage (T3-T4) compared to patients in earlier stages of the disease (T1-T2), thus suggesting a possible correlation between miR-223 and tumor volume (**Figure 15**).

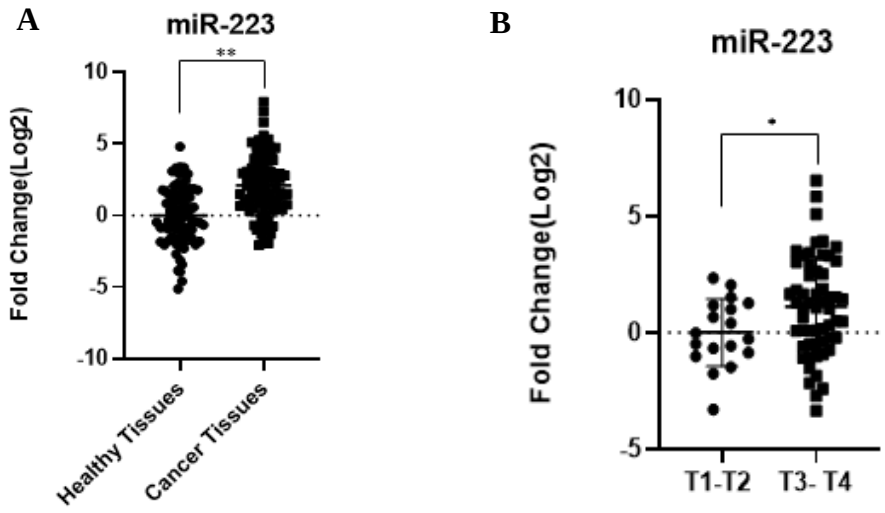


Figure 15 miR-223 Regulation in LSCC Tissues. (**A**) The expression levels of miR-223 in LSCC tissues (n= 83) compared to non-cancerous specimens (n= 83) and (**B**) miR-223 levels' comparison between advanced stages T3-T4 (n= 57) and early stages T1-T2 (n= 18). The *p*-value was calculated by t-test *$p<0.05$, **$p<0.005$.

3.2 Stable miR-223 Lentiviral Transduction in LSCC Cell Lines

The biological function of miR-223 in LSCC pathogenesis and metastasis occurrence was characterized *in vitro* by lentiviral transduction of HEp-2 and HNO-210 LSCC cell lines, using miR-223 mimic, miR-223 inhibitor or the corresponding negative controls (NC mimic and NC inhibitor). The best condition of transduction efficiency for any subset of transduced cells was obtained at 20 MOI (Multiplicity Of Infection, *i.e.* the ratio of the number of transducing lentiviral particles to the number of cells) (**Figure 16**).

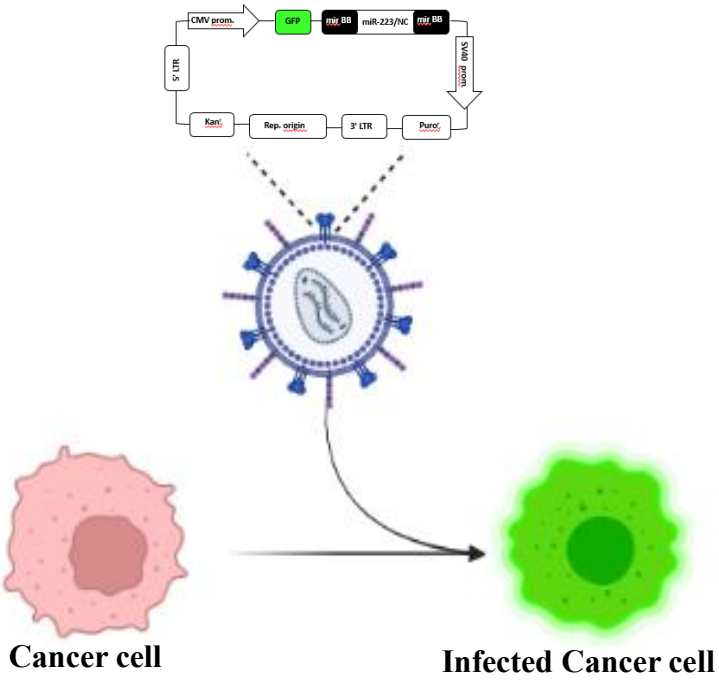


Figure 16 Graphical Representation of *in vitro* Transduction Mechanism. Cancer cells are infected with lentiviral vectors containing the insert that express a specific gene and a GFP reporter which will be visible as fluorescence in the infected cells.

The LSCC cellular systems selected for the present study were preliminary tested for Puromycin sensitivity. Cells were treated for 24, 48 and 72h with 10 different concentrations of Puromycin ranging from 0 to 4 µg/ml. Thereafter, HEp-2 and HNO-210 cells were processed for viability assay to select the lowest concentration able to kill 100% of cells. Based on our results, the minimal lethal concentration was 1.5 µg/ml for both cell lines (**Figure 17**).

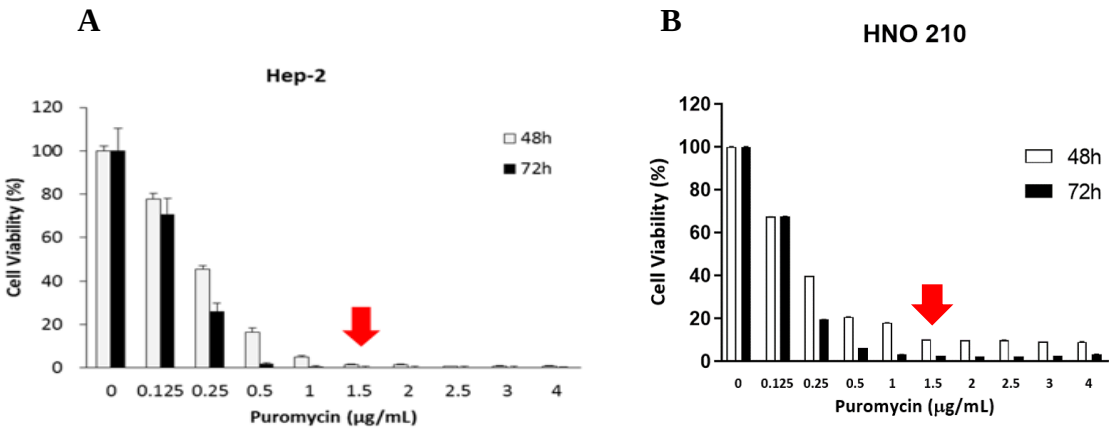


Figure 17 Antibiotic Killing Curve. HEp-2 (**A**) and HNO-210 (**B**) cell viability assay to select the minimal lethal concentration of puromycin. 10 different puromycin concentrations ranging from 0 to 4µg/ml were tested.

To induce a long-term stable expression, we transduced HEp-2 and HNO-210 cells with a viral-based system. Lentiviruses are a subgroup of retroviruses, which integrate foreign genetic material into the host genome. These viral vectors are genetically modified to make them unable to replicate, thus increasing their safety.

We designed two different lentiviral vectors, pLenti-III-miR-GFP and pLenti-III-miR-Off-GFP by integrating miR-223-3P mimic and miR-223-3P inhibitor sequences in lentiviral genome, respectively. The miRNAs sequences were obtained from miRbase. Other two vectors, corresponding to miR-223 mimic negative control (pLenti-III-miR-GFP-Blank) and miR-223 inhibitor negative control (pLenti-III-miR-Off-GFP-Blank), were instead developed by integrating specific random sequences into the lentiviral genome (**Figure 18**).

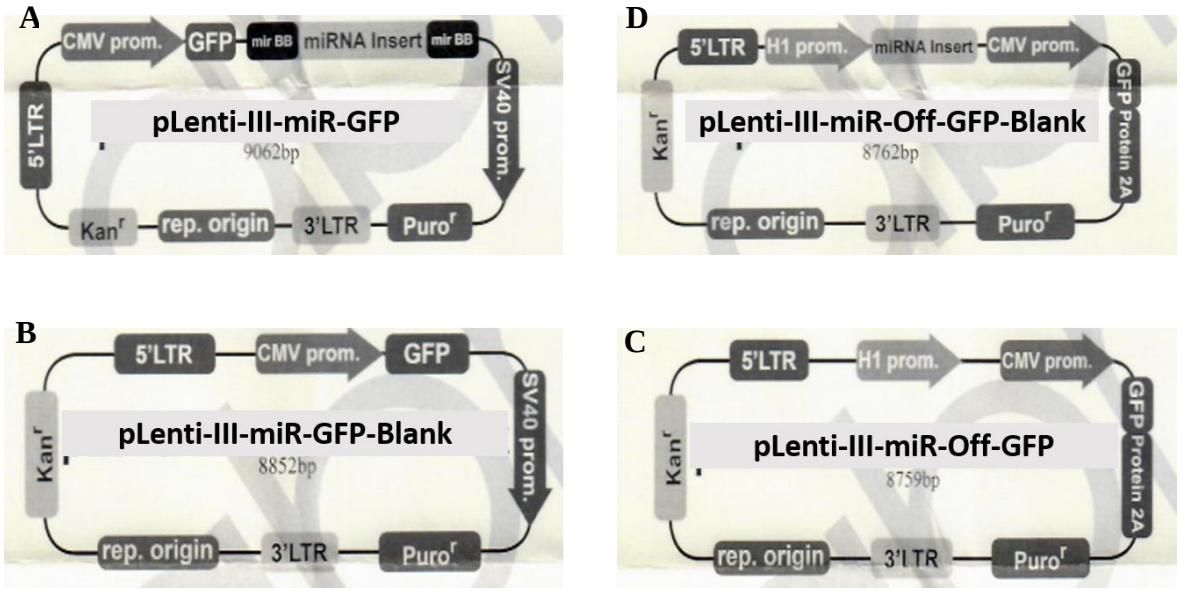


Figure 18 Illustration of Customized Lentiviral Vectors. Lentiviral vectors for (**A**) miR-223-3P mimic (pLenti-III-miR-GFP), (**B**) NC mimic (pLenti-III-miR-GFP-Blank), (**C**) miR-223-3P inhibitor (pLenti-III-miR-Off-GFP) and (**D**) NC inhibitor (pLenti-III-miR-Off-GFP-Blank).

After transduction, cells were selected in culture medium supplemented with 1.5 µg/ml Puromycin and monitored for transduction efficiency by fluorescence microscopy. The images were processed and cell fluorescence was measured using ImageJ Software. The results showed the transduction efficiency expressed as fluorescence intensity of infected cells compared to untreated ones, suggesting that the successful transfection of HEp-2 and HNO-210 cell lines provides a good preclinical model for the evaluation of miR-223 *in vitro* modulation (**Figure 19**).

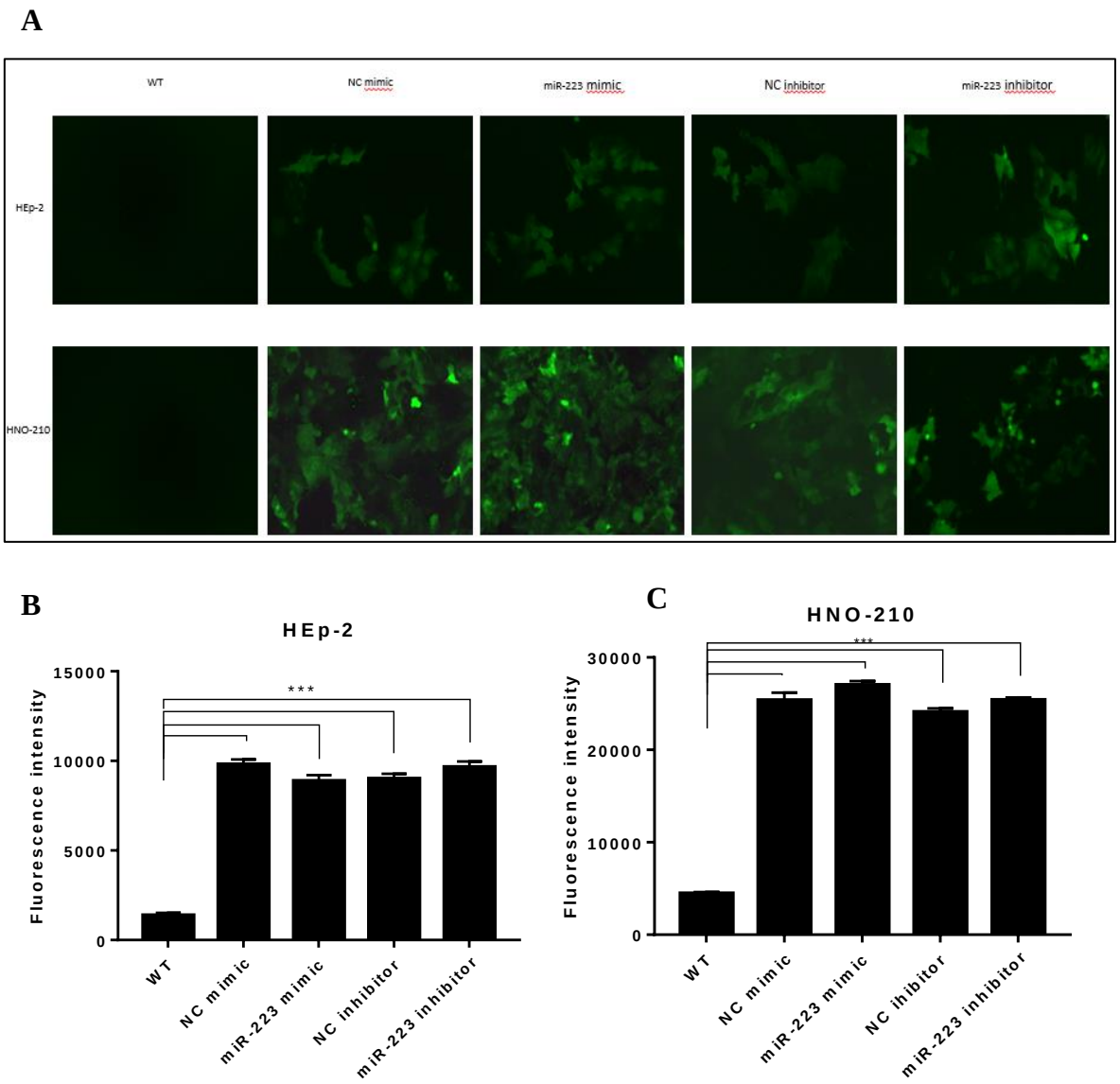


Figure 19 Intensity Fluorescence Evaluation for Monitoring Transduction Efficiency. HEp-2 and HNO-210 cells transduced with specific lentiviral vectors (NC mimic, miR-223 mimic, NC inhibitor, miR-223 inhibitor) were monitored at fluorescence microscope (**A**). Images (**B-C**) were analysed in comparison with WT (no transduced cells). The *p*-value was calculated by t-test ****p<0.001.*

Afterwards, we examined the transduction efficiency also evaluating miR-223 expression levels by quantitative RT-PCR in transduced HEp-2 and HNO-210 cells. On the basis of qRT-PCR data, we observed a significantly marked miR-223 expression in HEp-2 and HNO-210 cells transduced with miR-223 mimic compared to NC miR-223 (*p<0.001*), and

a less marked, but significant inhibition of miR-223 expression induced by miR-223 inhibitor transduction ($p<0.05$). Both HEp-2 and HNO-210 cells showed no significant modulation of miR-223 expression levels when compared to untreated cells or the corresponding negative control (**Figure 20**).

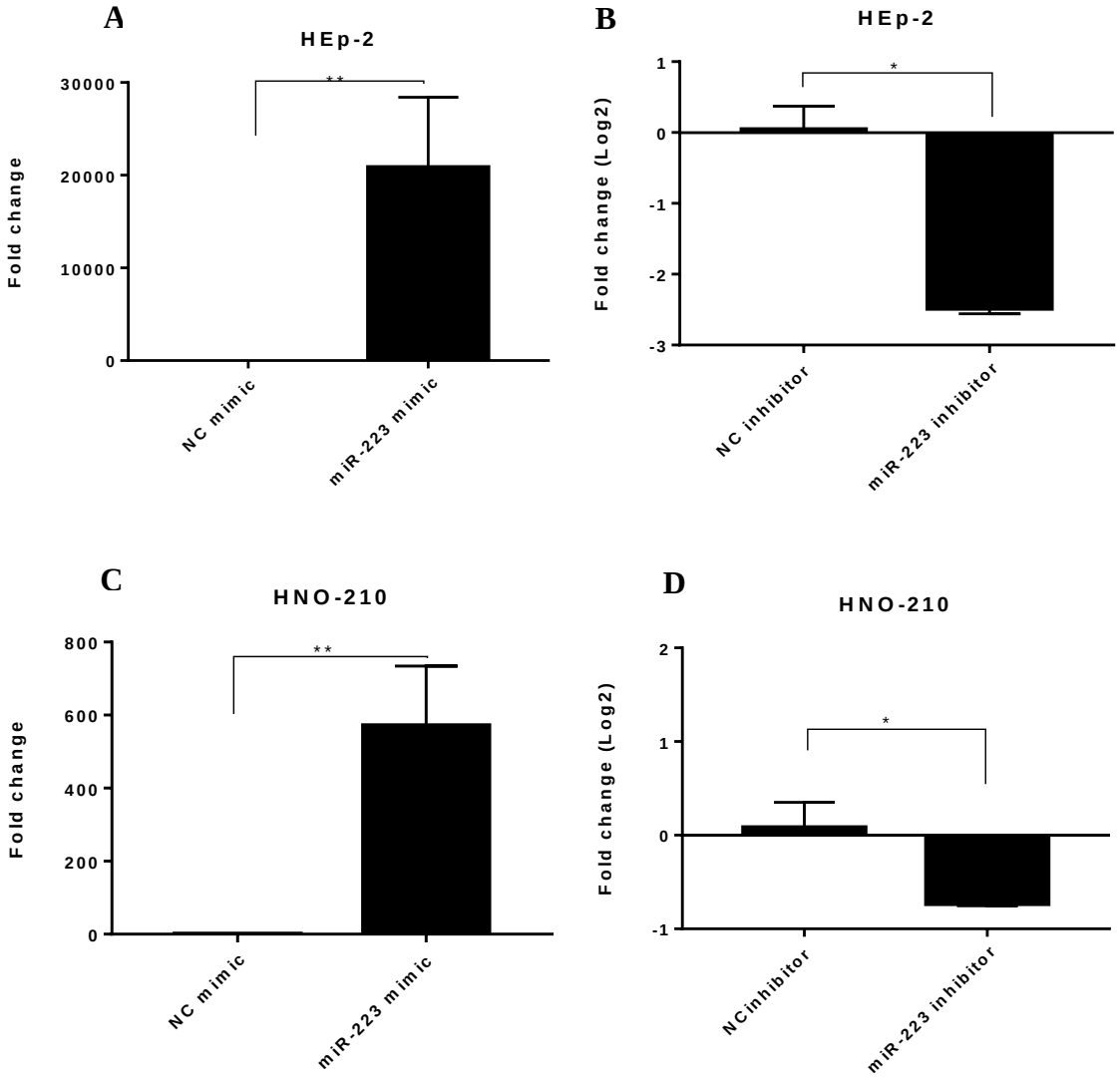


Figure 20 Stable Expression of miR-223 mimic and miR-223 inhibitor in LSCC Cell Lines. Relative expression levels of mir-223 in HEp-2 (**A-B**) and HNO-210 (**C-D**) cells after transduction with either miR-223 mimic or inhibitor, and corresponding NC. U6 snRNA was used as a reference gene. Error bars show mean ± SD. The *p*-value was calculated by t-test using each ΔCt values. *p <0.05, **p <0.001.

3.3 Anti-proliferative Effects of miR-223 inhibitor in LSCC Cells

Transduced HEp-2 and HNO-210 cells (NC mimic, miR-223 mimic, NC inhibitor, miR-223 inhibitor) were cultured and processed for cell viability evaluation after 24h, 48h, 72h and 96h by MTT assay. We observed a pronounced cell growth enhancement in both HEp-2 and HNO-210 cells transduced with miR-223 mimic, compared to the relative NC, at 96h ($p<0.05$) after seeding. On the other hand, miR-223 silencing resulted in significant cell growth inhibition at both 48h ($p<0.005$) and 72h ($p<0.05$) for HEp-2 cells and at 24h ($p<0.05$), 48h ($p<0.005$) and 72h ($p<0.005$) for HNO-210. In detail, miR-223 inhibitor caused a 10-20% decrease of cell viability for HEp-2 and 10%-40% for HNO-210 cells, outlining a global anti-proliferative effect (**Figure 21**).

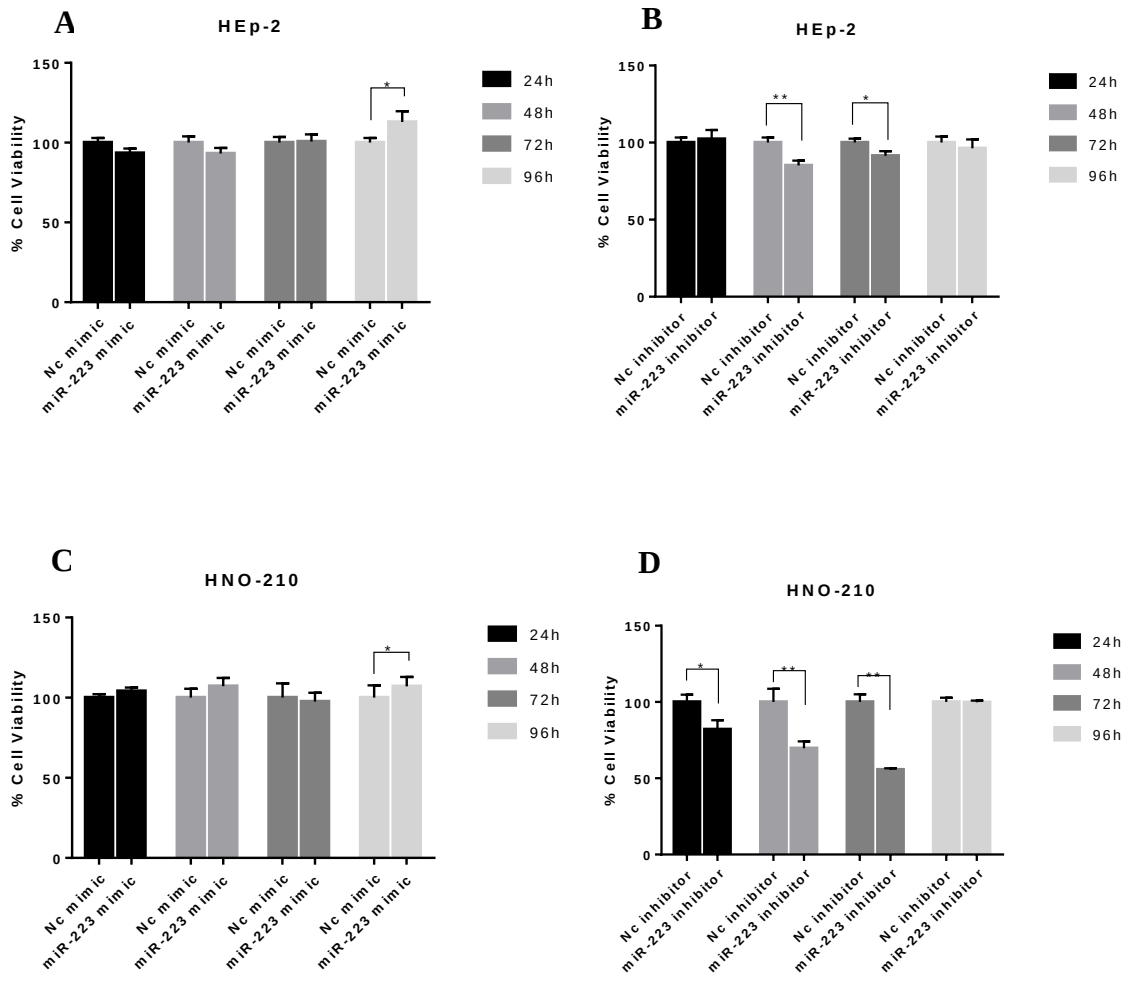
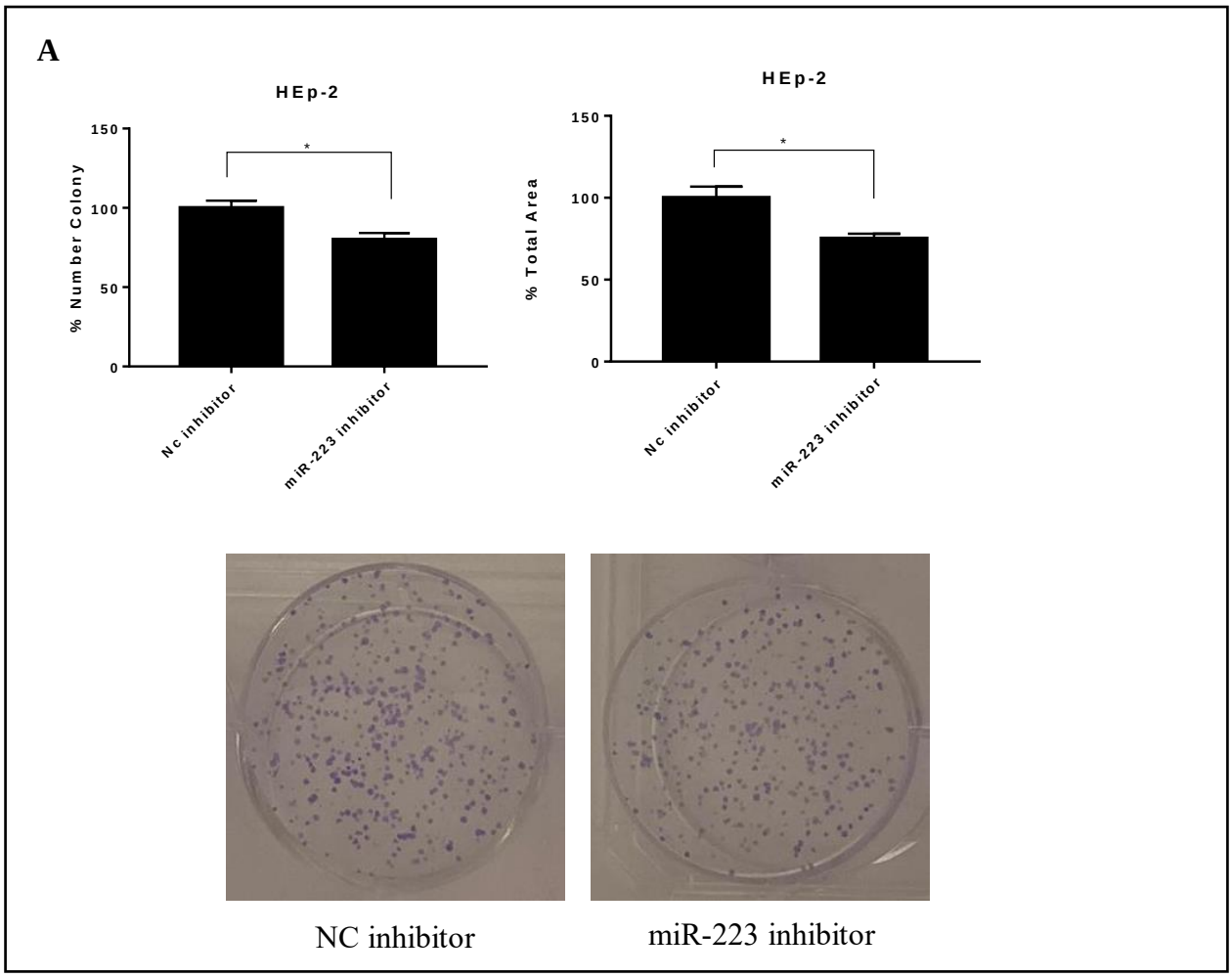


Figure 21 Ectopic miR-223 mimic and miR-223 inhibitor Expression Affects HEp-2 and HNO210 Cell Growth. Cell growth of HEp-2 and HNO210 cells, transduced with either miR-223 mimic (**A-C**) or inhibitor (**B-D**), was evaluated by MTT assay at 24, 48, 72 and 96h in comparison with the corresponding NC. Error bars show mean ± SD. The *p*-value was calculated by t-test * p <0.05, ** $p<0.005$.

To confirm these results, we also carried out a clonogenic assay for the evaluation of single cell ability to undergo unlimited division growing in colonies. The assay was performed on HEp-2 cells transduced, as above described, with miR-223 mimic, miR-223 inhibitor or their corresponding NC.

As expected, miR-223 inhibitor significantly limited the relative number (decrease of about 20%, *p<0.05*) and area (decrease of about 30%, *p<0.05*) of cell colonies over a period of 10 days, compared to the corresponding NC (**Figure 22 – Panel A**).



Simultaneously, an opposite effect was observed for miR-223 ectopic expression, which enhanced self-renewal capacity of HEp-2 cells. In detail, LSCC cells transduced with miR-223 mimic exhibited a significant increase of both colonies relative number (about 20%, *p<0.05*) and area (about 40%, *p<0.05*) when compared to the corresponding NC (**Figure 22 – Panel B**).

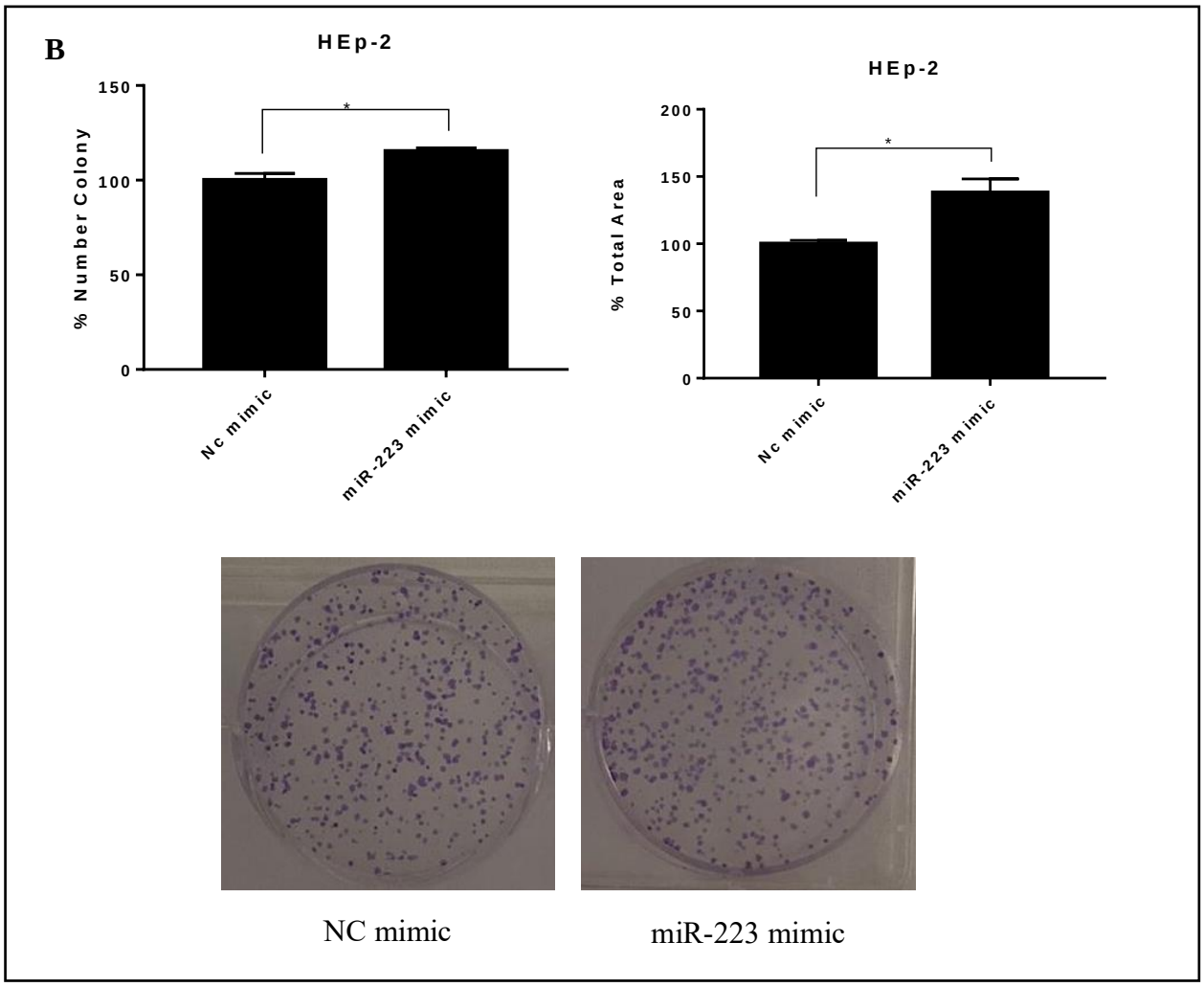


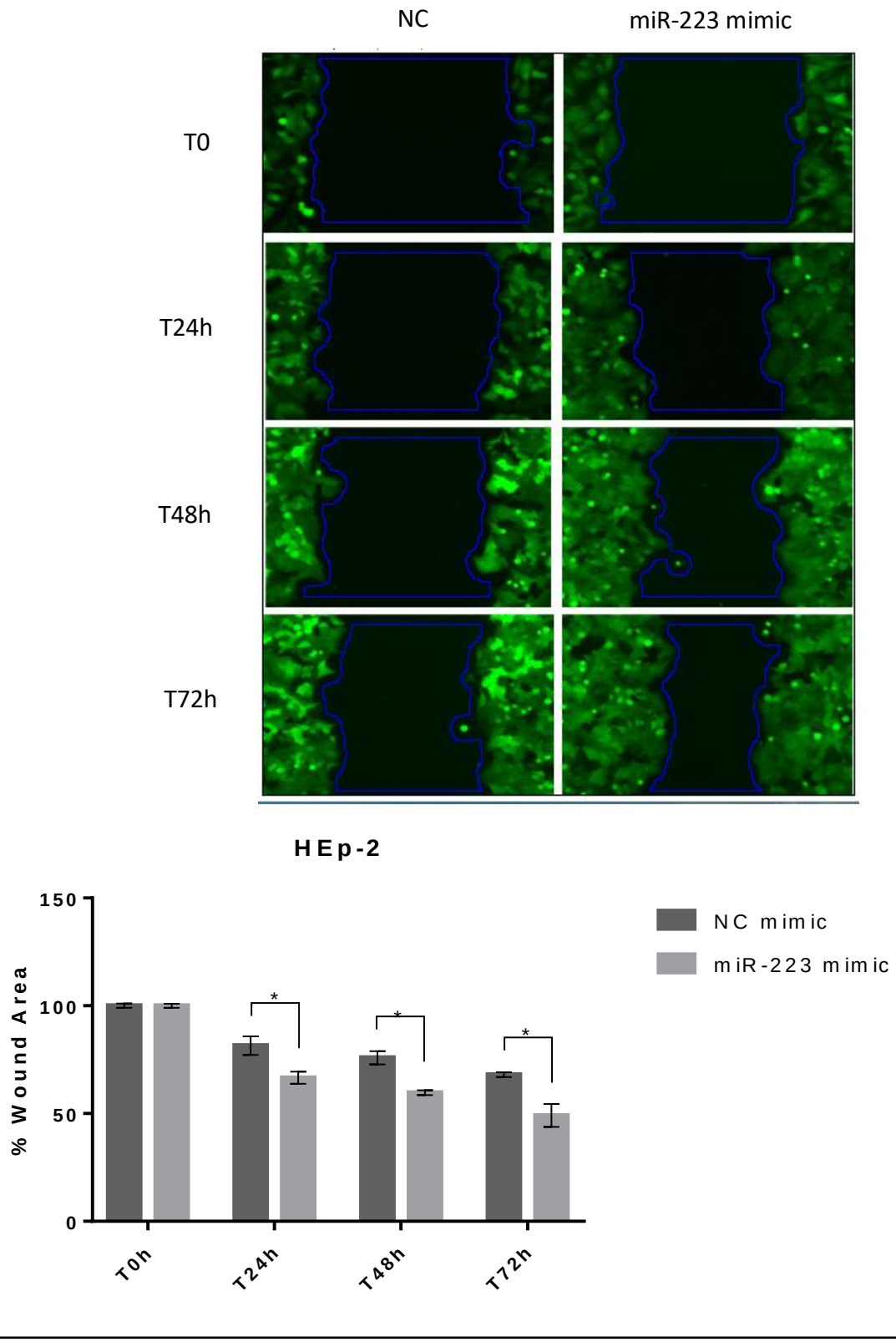
Figure 22 Clonogenic Assay. Clonogenic assay performed on HEp-2 cells transduced with lentiviruses expressing miR-223 inhibitor (**Panel A**) or miR-223 mimic (**Panel B**). Proliferating colonies after 10 days were fixed with 70% ethanol, stained with 0.25% crystal violet and analyzed. The data represent the number and area of colonies ± standard deviation (SD) of triplicates. The *p*-value was calculated by t-test **p* <0.05.

Collectively, these data suggested that miR-223 inhibitor exerts a powerful growth inhibition, affecting both the viability and the clonogenic potential of LSCC cells.

3.4 Mir-223 Silencing Counteracts Migratory Behaviour of LSCC Cells

In the same experimental conditions, we also evaluated how miR-223 aberrant expression affected tumor migratory process, performing a wound-healing assay. Interestingly, cells transduced with miR-223 inhibitor showed a significant decrease of wound closure rate in comparison with NC inhibitor, particularly at 72h ($p<0.05$), thus demonstrating a miR-223-dependent attenuation of migratory phenotype and, likely, a counteraction of metastatic potential. On the other hand, HEp-2 cells transduced with miR-223 mimic displayed a mighty increase of the migratory capacity at 24, 48 and 72h ($p<0.05$), when compared to the corresponding NC (**Figure 23**).

A


NC
miR-223 mimic
T0
T24h
T48h
T72h
HEp-2
% Wound Area
150
100
50
0
T0h
T24h
T48h
T72h
NC mimic
miR-223 mimic
*
*
*

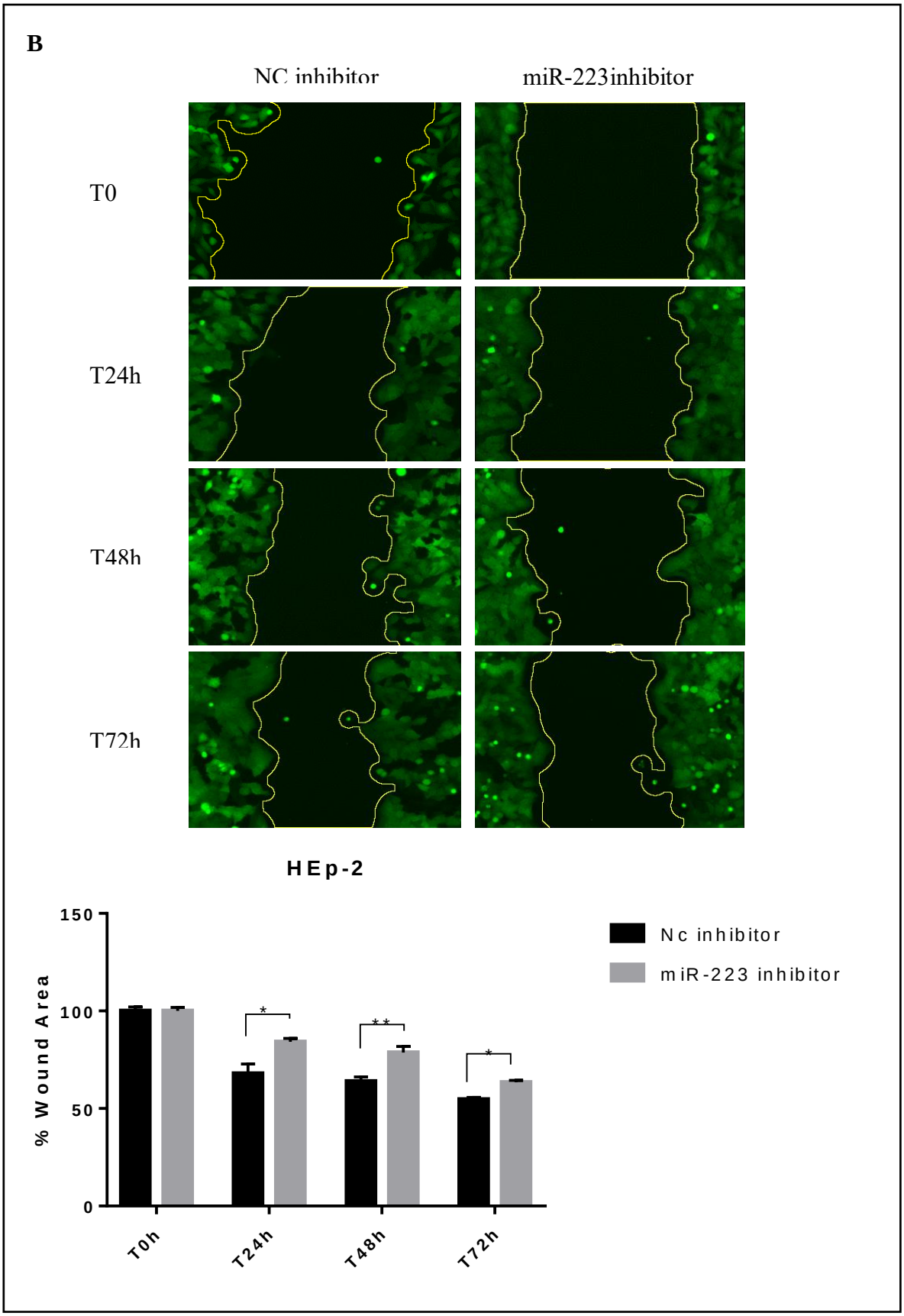


Figure 23 Effects of Ectopic Expression of miR-223 inhibitor on Migration of HEp-2 Cells. Confluent monolayer of HEp-2 cells, transduced with miR-223 mimic, miR.223 inhibitor and

corresponding NC, were scratched to create an artificial wound. In **Panel A** miR-223 mimic influence on cell migratory capacity, was monitored by microscopy at T0 and after 24, 48 and 72h and analysed in comparison with NC mimic, objectively with ImageJ. At the same way in **Panel B** HEp-2 cells transduced with miR-223 inhibitor were assayed in respect to NC inhibitor. Error bars show mean ± SD. The *p*-value was calculated by t-test. **p* <0.05, ***p* <0.005

3.5 Bioinformatic Analysis of Potential Cancer-related miR-223 Targets

Taking into account the opposite *in vitro* effects of miR-223 mimic and miR-223 inhibitor, in particular the observed results on migration and tumorigenicity, as well as our clinical evidence on the correlation between tumor stage and miR-223 expression in LSCC cancer patients, we hypothesized a tumor promoting function for miR-223. Thus, in order to better understand the molecular mechanism of its oncogenic role, we focused on the possible modulation of the main tumor-suppressor targets. For this purpose, we queried four different algorithms of *in silico* miRNA target prediction (TargetScan (version 7.1), DIANA-microT-CDS, (version 5.0), miRANDA-mirSVR (released 2010) and miRmap) and all bioinformatic platforms displayed the direct targeting of MTSS1 (Metastasis suppressor protein 1). This targeting, due to complementary interaction involving 169-176 positions of 3' UTR region in MTSS1 mRNA, was predicted with a score of 79 (**Figure 24**).

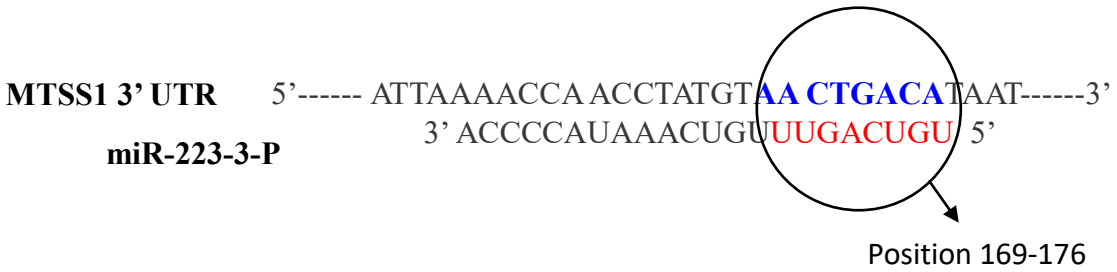


Figure 24 Putative Interaction Model between miR-223 and MTSS1 Gene. In the interaction model between miR-223 and MTSS1 mRNA, the predicted binding site is located at position 169-176.

MTSS1 may be related to cancer progression or tumor metastasis in a variety of organs, most likely through an interaction with the actin cytoskeleton (Xie F, 2011). Of note, there are also other possible direct or indirect miR-223 targets able to contribute to EMT, migration, invasion, inflammation and subsequent metastasis formation. In particular, a number of evidence in literature already argued the role played by miR-223 up-regulation in EMT process in cancer (Ma J, 2015). For these reasons, we decided to analyse with DIANA-miRPath algorithm, all the predicted and experimentally validated miR-223 targets involved in cancer-related molecular pathways. Among the most significant targets we found ACVR2A and IL6ST, involved in cytokine production and inflammation, as well as a large number of transcriptional factors – PAX5, BMP2K, RELA, FOXO1 and HHEX- able to regulate tumor progression (**Table 5**).

Cytokine-Cytokine Receptor Interaction	**Transcriptional Misregulation in Cancer**
ACVR2A	PAX5
IL6ST	BMP2K
	RELA
	FOXO1
	HHEX

Table 5 *in silico* Targets Prediction and Pathway Enrichment Analysis

IV DISCUSSION

Laryngeal cancer is one of the most common malignant forms of head and neck district neoplasms. During the past three decades, its incidence and prevalence have increased by 12.0% and 23.8% respectively, with more than 180,000 new cases reported yearly worldwide, ranking as the 22nd in incidence and the 18th in prevalence and mortality (Nocini R, 2020). T he most common histological type is classified as laryngeal squamous cell carcinoma (LSCC), accounting over 90% of the malignancy (Almadori G, 2005).

Among all the treatments available for LSCC, the options for advanced stages usually include a combination of chemotherapy and radiotherapy, while surgical resection and radiation therapy are currently used for early-stage cancers. Compared to the past, LSCC surgery is less invasive, tending to organ preservation and used only for rescue treatment or for injuries with extra-laryngeal extension or cartilage destruction. However, due the absence of specific symptoms and reliable markers, LSCC is usually diagnosed in advanced stages, resulting in delayed treatment, high frequency of tumor recurrence, metastasis and worse prognosis.

miRNA identification into the bloodstream or other biological fluids, has generated great interest for the potential use as biomarkers. Circulating microRNAs, are a new class of gene regulators, whose role in cancer onset and progression has been deepened, opening new opportunities for therapeutic application. They belong to the family of small non-coding RNAs, molecules of ~24 nt that can inhibit mRNA translation and/or negatively regulate its stability. In the last years, an increasingly number of dysregulated miRNAs in plasma or serum, have been considered as a novel hallmark of cancer.

A large body of evidence showed aberrant miRNA regulation in LSCC tissues and/or plasma (Ricciardiello F, 2017) (Cao P, 2013) (Ayaz L, 2013), underlining a strong relation between the expression levels and the main processes underlying tumor initiation and progression, such as proliferation, migration, invasion, metastasis, tumor infiltration, and disease relapse. Based on the modulation trend and on bioinformatic analysis of the putative targets, several reports have delineated a possible oncogenic or tumor-suppressive role for several miRNAs in LSCC pathogenesis, however the molecular mechanism is still unclear and further insights are required for their employ as diagnostic, prognostic and therapeutic tools.

In this scenario, the objective of the present paper has been to determine a miRNA signature for the definition of LSCC diagnosis and prognosis, as well as to characterize *in vitro* the functional role of miR-223, which is one of the most interesting microRNAs selected as biomarker candidate.

First of all, we carried out a high-throughput PCR array analysis on 45 patients, including 22 with lymph node metastases (N^+) and 23 without (N^-), compared with 23 healthy volunteers. MiRNA profiling allowed us to detect 81 dysregulated miRNAs (**Figure 11**) and, among them, we observed the significant up-regulation of miR-532, miR-93, miR-451, miR-140, miR-223, miR-16, miR-20b, miR-29a, miR-132, miR-25, miR-20a, and the significant down-regulation of miR-95, miR-150, miR-891a, miR-331 and miR-374 (**Table 2**). Based on the microarray analysis and on the further validation data derived from qRT-PCR performed in a small cohort of samples (**Figure 12**), we focused on miR-532, miR-93 and miR-223 as up-modulated candidates. Consistently with the microarray results, we confirmed their significant up-regulation on an increasingly large cohort of LSCC patients (75 patients, divided into 32 N^+ and 43 N^-) (**Figure 13**). The selected miRNAs were also investigated for their diagnostic value using ROC curve analysis (**Figure 14**). As a result, a moderate diagnostic potential was revealed, together with a low specificity. This outcome is certainly affected by the low sample size; therefore, the progressive expansion of study population will be indispensable to make a definitive elucidation. Interestingly, miR-532, miR-93 and miR-223 diagnostic potential for LSCC detection seemed to be not correlated with clinical features like sex, age, and lymph node metastasis involvement, but both miR-532 and miR-93 overexpression was more prominent in T1-T2 stages, suggesting their ability as early diagnostic markers (**Table 4**).

miR-93, miR-223 and miR-532 are involved in carcinogenesis, progression and metastasization of different neoplasms and, in particular, many studies dissected the role of miR-223 and miR-93 in head and neck squamous cell carcinoma (HNCCS).

miR-93 overexpression has been detected in LSCC tissues (Cao P, 2013) and it oncogenic function in LSCC cells has been demonstrated affecting proliferation, migration, invasion and apoptosis (Xiao X, 2015). The high miR-93 expression has been also observed in HNSCC tissues and correlated with cancer progression, metastasis and poor prognosis (Li G, 2015). As well, a high-throughput qRT-PCR array for miRNA profiling in LSCC patients, showed a significant up-regulation of plasma miR-93 levels in cancer group compared to control ones (Ayaz L, 2013). Regarding miR-223, its up-regulation in oral squamous cell carcinoma (OSCC) tissues and cell lines increased cell growth and migration and suppressed apoptosis through direct targeting tumor suppressor FBXW7 (Jiang L, 2019). In HNSCC tissues, it has been reported a significant correlation between miR-223 high expression and neutrophil infiltration. Moreover, in HNSCC cells, miR-223 ectopic expression increased proliferation, apoptosis and resistance to Cetuximab, also inducing pERK2, pAKT and AKT

expression and angiogenesis inhibition (Bozec A, 2017). Both miR-93 and miR-223 have been found up-regulated in gastric cancer tissues where the modulation was associated with the metastasis occurrence. In detail, *in vitro* and *in vivo* assays showed that miR-93 induced gastric cancer cell migration, invasion and metastasis by targeting IFNAR1, which is a negative regulator of STAT3/MMP9 axis (Ma DH, 2017); likewise, miR-223, whose expression was induced by a transcription factor Twist, acted directly targeting the tumor-suppressor EPB41L3 (Li X, 2011). Moreover, in pancreatic cancer, the metastatic process was also induced by miR-223-dependent EMT stimulation via Fbw7 down-regulation and subsequent NOTCH1 up-regulation (Ma J, 2015). Cancer-related miR-532 function has been characterized in several neoplasms excepting LSCC, hence further evaluation of both expression levels and biological functions could be remarkable.

Based on these findings, the high expression levels of miR-93, miR-223 and miR-532 represent an important starting point to better investigate their role in carcinogenesis and their powerful potential for LSCC minimally invasive diagnosis and/or prognosis.

At this point we focused our attention on miR-223, which already exhibited a strong deregulation in LSCC cancer tissues too, as report in a previous microarray analysis carried out by our team (Ricciardiello F, 2017). In particular, we analysed miR-223 tissue expression data highlighting a significant up-regulation in patients with advanced cancer (T3-T4) compared to patients in earlier stages of the disease (T1-T), suggesting a possible correlation between miR-223 levels and tumor extension (**Figure 15**).

We investigated the molecular effects of miR-223 deregulation using two *in vitro* models represented by HEp-2 and HNO-210 LSCC cell lines transduced with third generation lentiviral vectors for stable expression of miR-223 mimic, miR-223 inhibitor, or their corresponding negative controls (*i.e.* oligonucleotides with specific random sequences). Lentiviral transduction can in fact efficiently deliver nucleic acids into *in vitro* cultured cells. Transduction efficiency was firstly examined by quantifying the relative fluorescence intensity of GFP (**Figure 19**) and then by evaluating miR-223 expression levels in qRT-PCR (**Figure 20**). The results showed a marked miR-223 overexpression in HEp-2 and HNO-210 cells transduced with miR-223 mimic compared to NC miR-223. On the contrary, miR-223 inhibitor induced a slightly lower knocking-down of miR-223 respect to the corresponding NC. These data, together with the high fluorescence intensity of all transduced cells in comparison with *wild type* LSCC cell lines, suggested that the successful transfection of HEp-2 and HNO-210 cell lines provides a good

preclinical model for the *in vitro* evaluation of biological effects resulting from miR-223 modulation.

Analysing the pro-tumorigenic effect of miR-223 on both HEp-2 and HNO-210 cell lines, we clearly demonstrated the promoting role of miR-223 ectopic expression on cell growth, mainly at 96h. On the other hand, the anti-proliferative efficacy of miR-223 inhibitor resulted significant at 48h and 72h for HEp-2 cells, while HNO-210 significantly decreased cell viability already after 24h and more markedly at 48h and 72h (**Figure 21**). To confirm these results, we also evaluated the long-term survival effects induced by miR-223 mimic and inhibitor, by clonogenic assay. As expected, miR-223 ectopic expression enhanced self-renewal capacity of HEp-2 cells; conversely, miR-223 inhibitor significantly limited number and area of cell colonies (**Figure 22**). Finally, in order to expand our investigation *in vitro* on the miR-223 functional role in pro-metastatic processes, we performed a wound-healing assay on transduced HEp-2 cells. Interestingly, we observed the anti-migratory activity of miR-223 inhibitor, mainly at 72h, and the increased cell migration at 24, 48 and 72h for HEp-2 transduced with miR-223 mimic (**Figure 23**).

It has been recently found that miR-223 promotes the development of invasive behaviour of different solid tumors through the EMT, particularly in gastrointestinal neoplasms. To better understand the molecular basis underlying the pro-tumorigenic and pro-metastatic effect of miR-223, we focused on the modulation of the main tumor-suppressor targets. For this purpose, we queried four different algorithms of *in silico* miRNA target prediction (TargetScan (version 7.1), DIANA-microT-CDS, (version 5.0), miRANDA-mirSVR (released 2010) and miRmap). All bioinformatic platforms displayed the direct targeting of MTSS1 (Metastasis suppressor protein 1), a metastasis suppressor down-regulated in many type of cancers (Xie F, 2011). Its targeting was predicted with a score of 79, and the complementary involves the site 169-176 of MTSS1 mRNA 3' UTR region (**Figure 24**).

Moreover, we used DIANA-mirPath algorithm for miRNA pathways enrichment analysis. Through a cross-search by three different databases, the latter revealed the possible miR-223-dependent transcriptional regulation of both cancer-related transcriptional factors and cytokine/cytokine receptor interaction mediators. Among the most significant genes, stand out IL6ST, ACVR2A, PAX5, BMP2K, RELA, FOXO1 and HHEX (**Table 5**), playing well known roles in inflammation, proliferation, migration, apoptosis and angiogenesis in different type of solid tumors.

ACVR2A and IL6ST participate to inflammatory pathways. ACVR2A is believed to be a tumor suppressor that inhibits cell growth and differentiation and its inactivation has been

proposed as a key factor in colorectal cancer development (Ballikaya S, 2014). Similarly, IL6ST was found downregulated in triple negative breast cancer, with higher expression corresponding to better prognosis (Jia R, 2021).

PAX5, BMP2K, RELA, FOXO1 and HHEX are transcriptional factors regulating tumor-related processes. In breast cancer cells, PAX-5 supresses cell proliferation and modulates epithelial-mesenchymal/mesenchymal-epithelial (EMT/MET) transition. More precisely, PAX-5 suppresses breast cancer cell migration, invasion and tumor spheroid formation (Benzina S, 2017). The reduced expression levels of BMP2K (Song X, 2020), RELA (Ricca A, 2001) and HHEX (Li X, 2021), are also associated to the aggressive features of various cancer types, affecting migration, invasion and apoptosis. FOXO1 is a key regulator of a broad range of cancer-related functions, including cellular differentiation, apoptosis, cell cycle arrest and DNA damage. Its onco-suppressive role has been reported in many type of human cancer, particularly for its involvement in PI3K/AKT pathway (Gheghiani L, 2020) (Zhang X, 2011).

Collectively, our findings suggest that miR-223 could target and regulate multiple factors playing a crucial role in pro-metastatic pathways; conversely, miR-223 inhibitor, able to reduce LSCC cell migratory capacity, could represent a novel therapeutic tool against primary, advanced and metastatic LSCCs.

V CONCLUSIONS AND FUTURE PERSPECTIVES

LSCC is characterized by local growth and diffusion in neighbouring lymph nodes, although it frequently develops distant metastases via bloodstream. The diagnostic process and the correct therapeutic planning represent the main challenge to maximize the possibility of complete disease resolution and patients' quality of life improvement, trying to preserve laryngeal function. Therefore, successful clinical management of LSCC patients is strictly linked to the identification of reliable diagnostic and prognostic biomarkers. This aim could be more easily achieved through the characterization of both biological and molecular factors underlying this disease.

Presently, the advancements in molecular and genetic technologies allowed the identification of a plethora of molecular factors endowed with diagnostic and/or prognostic role, showing their biomedical potential for the application in personalized medicine and treatment monitoring of LSCC patients. Within the broad variety of these molecules, a central role seems to be played by non-coding RNAs. In the present study, we have delineated, for LSCC cancer patients, a possible miRNA signature for the early diagnosis of the disease. As well, we demonstrated the *in vitro* oncogenic potential of miR-223, displaying its pro-tumorigenic and pro-metastatic effects on LSCC cell lines. Analysing the putative miR-223 targets, we assumed that it could orchestrate the regulation of multiple cancer-related processes, such as inflammation, angiogenesis, apoptosis, proliferation, EMT, migration and invasion, confirming the *in vitro* observation and suggesting its promising prognostic function. Moreover, the *in vitro* inhibitory effect of miR-223 on both proliferative and migratory capabilities of LSCC cell lines, encourages the possibility to deepen the molecular mechanisms to develop novel therapeutic opportunities based on the use of short single-stranded oligonucleotides acting as nc-RNA antagonists in cancer.

These findings, together with the evidence in literature, provide the rationale for further experiments aimed to better understand the molecular bases of miR-223 oncogenic role, assessing its involvement in EMT and metastatic processes, as well as the anti-tumor effect of its inhibitor. The future advancements of the present project will be oriented towards the characterization of the expression profile of the main genes that take part in miR-223-related cellular processes. In addition, *in vitro* studies of metabolomic NMR analysis and direct gene profiling of over 700 cancer-related genes by NanoString digital technology are currently ongoing.

VI MATERIALS AND METHODS

6.1 Clinical Samples

Blood samples were collected from LSCC patients and healthy donors, enrolled at the Ear, Nose and Throat Division of the University of Naples -Federico II-, the University of Campania -L.Vanvitelli-, Monaldi and Antonio Cardarelli Hospitals. Informed consent was obtained from all patients.

The serum collection tube including blood was centrifuged at 3,000 rpm, at room temperature (R.T.) for 5 min. The supernatant was collected as serum into a 2 mL cryotube in draft chamber and stocked in -80 °C freezer until the extraction of miRNA.

Forty-five LSCC sera from 23 patients suffering from lymph node metastases (N^+), 22 patients without lymph node involvement (N^-), and 23 sera samples collected from healthy donors were enrolled for the Microarray study. 75 LSCC blood samples ($32N^+$ and $43N^-$) and 29 normal blood samples, including all subjects already joined in the preliminary screening, were used for the validation set.

6.2 Cell Cultures

HEp-2 LSCC cell line, obtained from the ATCC, was grown in MEM medium, containing L-glutamine (Gibco®, Life Technologies, Carlsbad, CA), supplemented with heat-inactivated 10% FBS (Lonza Group Ltd., Switzerland), 20 mM HEPES, 1% MEM non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA) and incubated at 37 °C in a 5% CO_2 atmosphere.

HNO-210 LSCC cell line, purchased from CLS Cell Lines Service GmbH, was grown in DMEM medium, containing L-glutamine (Gibco®, Life Technologies, Carlsbad, CA), supplemented with heat-inactivated 10% FBS (Lonza Group Ltd., Switzerland), 20 mM HEPES, 1% MEM non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA) and incubated at 37 °C in a 5% CO_2 atmosphere.

6.3 RNA Extraction

Total RNA extraction from laryngeal cancer cell lines was performed using mirVana™ PARIS™ Kit (Ambion) according to the manufacturer's protocol. The integrity, quality (Abs. 260 nm/230 nm and 260 nm/280 nm) and quantity of total RNA were assessed by the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA samples were stored in -80 °C freezer until further processing.

Serum miRNA isolation was carried out using TaqMan™ miRNA ABC Purification Kit - Human Panel A (Applied Biosystems), according to the manufacturer's manual. In this extraction procedure, 1 µL of 1 nM cel-miR-39-3p mimic (mirVana® miRNA mimics, Ambion) was added into 50 µL of serum as an external control. The purified serum miRNAs were stored in -80 °C freezer until the next experiment without measurement of purity and concentration of the extraction because of undetectable low miRNAs quantity.

6.4 Microarray Screening Assay

Serum miRNAs purified from 45 LSCC patients and 23 healthy donors were enrolled for miRNAs screening assay. The specimens of LSCC cohort were divided in two groups, *i.e.* 22 lymph node metastasis positive (N^+) and 23 negative (N^-) serum samples. For reverse transcription, an equal amount of serum miRNAs from different patients or donors, were mixed into 5 pools for each group (**Table 6**).

	LSCC Patients		***Healthy Donors***
Pool	***N^+***	***N^-***	
1	5	5	5
2	5	5	5
3	4	5	5
4	4	4	4
5	4	4	4

Table 6 Number of Samples from LSCC Patients or Healthy Donors for Each Pool Used in miRNAs Screening

Starting from each RNA pool, cDNA was synthesized with Megaplex RT Primers, Human Pool A v2.1 (Applied Biosystems, California, USA) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, California, USA), according to manufacturer's instructions. Human Pool A v2.1 contains RT primers for 377 unique microRNAs and 4 controls. Subsequently, cDNA was pre-amplified with Megaplex PreAmp Primers Human Pool A v2.1 (Applied Biosystems, California, USA) and TaqMan PreAmp Master Mix (Applied Biosystems, California, USA).

The miRNA expression profiling was finally performed using TaqMan Array Human MicroRNA A Cards v2.0 (Applied Biosystems, California, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, California, USA), following manufacturer's manuals. The assay was run on Viia 7 real-time PCR system (Applied Biosystems, California, USA).

The Ct value was determined using Viia7 software (Applied Biosystems, California, USA) and setting a threshold of 0.2. For normalization of miRNAs expression, NormFinder analysis was conducted with corresponding R software available online (http://moma.dk/normfinder-software), and miR-222, which was the most stably expressed among all pools, was selected as a reference miRNA. Thus, ΔCt was obtained using the formula: ΔCt = Ct target miRNA - Ct miR-222, and the relative miRNA expression was calculated with the ΔΔCt method (ΔΔCt = ΔCt interest group – ΔCt control group). Fold change was calculated with the equation $2^{-\Delta\Delta Ct}$ method and converted to logarithm (Log2).

6.5 Design of Lentiviral Vectors

Custom third-generation Lentiviral vectors were provided by abm (Applied Biological Materials). Lentiviral vectors for miR-223-3P mimic (pLenti-III-miR-GFP) and miR-223-3P inhibitor (pLenti-III-miR-Off-GFP) were developed by using sequences from miRbase: 5'-UGUCAGUUUGUCAAAUACCCCA-3′ - miR-223 mimic sequuence - and 5'-CAGUACUUUUGUGUAGUACAA-3′ - miR-223 inhibitor sequence. Two other vectors containing specific random sequences - pLenti-III-miR-GFP-Blank and pLenti-III-miR-Off-GFP-Blank - were constructed as the corresponding negative controls. All the viruses contain a GFP reporter for monitoring transduction efficiency and two selectable markers conferring resistance to kanamycin and puromycin, for selecting infected host cells. They were purified by HPLC and viral titer were evaluated by qPCR.

6.6 *in vitro* Transduction of LSCC Cell Lines

The LSCC cell lines were seeded 24 hours before transduction and incubated at 37 °C in a 5% CO_2 atmosphere. In detail, HEp-2 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well in MEM medium without antibiotics, while HNO-210 were seeded in 48-well plates at a density of $45x10^3$ cells/well in DMEM medium without antibiotics.
The day after, concentrated lentiviruses were added to the medium with 8 µg/ml polybrene. 20 MOI (Multiplicity Of Infection) was the used ratio of transducing lentiviral particles per cell. 24 hours after lentiviruses infection, cell transduction efficiency was evaluated by observing the percentage of GFP^+ cells using microscope (EVOS® FL Cell Imaging System) (Life Technologies). Cell confluence was monitored in the following days and the scale up started when cells reached about 90% of confluence, from 96-well or 48-well plates to 6-well plates. In 6-well plates, about 7 days post-infection, 1.5 µg/ml of puromycin was added to the medium to start the selection of transduced cells.
The optimal concentration of puromycin, *i.e.* the minimal lethal concentration that kills 100% of cells, was preliminary determined performing a killing curve. HEp-2 and HNO-210 cells were seeded into 96-well plates at a density of 3000 and 12500 cells/well respectively and incubated overnight at 37 °C in 5% CO_2. The day after, complete growth medium was replaced with selection medium supplemented with a range of antibiotic concentrations (0.125-0.250-0.5-1-1.5-2-2.5-3-4 µg/ml) except for untreated control cells. After 24, 48 and 72 hours the percentage of surviving cells was evaluated through viability assay.

6.7 Real-Time quantitative PCR

Total RNA from LSCC cell lines was extracted as described above. Oligo-dT-primed cDNA was obtained using TaqMan™ MicroRNA Reverse Transcription Kit. TaqMan™ Fast Advanced Master Mix (Applied Biosystems, California, USA) was used to detect and quantify mature miR-223, according to the manufacturer's instructions by Real-time PCR Viia7 (Applied Biosystems, California, USA). MiR-223 expression was normalized with U6 snRNA (Ambion, Life Technologies, California, USA).
For validation of miRNA candidates, serum miRNAs samples, originated from both LSCC patients and healthy donors, were tested by qRT-PCR. cDNA synthesis was performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, California, USA),

according to manufacturer's manual. In order to detect miR-223, miR-93 and miR-532 quantitative PCR was performed using TaqMan Fast Universal PCR Master Mix (2X) no AmpErase UNG (Applied Biosystems, California, USA) under the control of ViiA 7 Real-Time PCR System (Applied Biosystems, California, USA). MiR-223, miR-93 and miR-532 expression was normalized with the external control cel-miR-39-3p.

Comparative real-time PCR (RT-PCR) was performed in triplicate, including no template controls, and relative expression was calculated using the comparative cross-threshold (Ct) method.

Cycle threshold (Ct) value was calculated using ViiA™ 7 Software (Applied Biosystems) with threshold set to 0.2. Subsequently, for normalization of target gene expression level, ΔCt was derived by formula: Ct of target gene - Ct of reference gene such as U6 snRNA (endogenous miRNA reference) and cel-miR-39-3p (exogenous miRNA reference). ΔΔCt was calculated by formula: ΔCt of interest group - ΔCt of control group, and then $2^{-\Delta\Delta Ct}$ was derived as a fold change (FC) of target gene expression.

6.8 Cell Viability Assay

Transduced HEp-2 and HNO-210 LSCC cells were seeded into 96-well plates at a density of 3000 and 12500 cells/well respectively and incubated at 37 °C in 5% CO_2. After 24, 48, 72, and 96h, the cells were stained using MTT reagent (Sigma-Aldrich) for 4 h. Acidified isopropanol was added to dissolve MTT into each well and mixed for 20 min by shaking. Absorbance 570 nm was measured by iMark™ Microplate Absorbance Reader (Bio Rad). Each experiment was performed in triplicate and data were expressed as mean ± SD.

6.9 Clonogenic Assay

For colony formation assays, transduced HEp-2 LSCC cells were seeded into 6-well plates at a density of 900 cells/well and cultured for 10 days.

After fixing with cold 70% ethanol and staining with 0.25% crystal violet, proliferating colonies were photographed. The colony counts were measured by ImageJ analysis (NIH

Bethesda, MD). The data represent the number and area of colonies ± standard error of the mean (SEM) of triplicates, and the experiments were repeated three times.

6.10 Wound Healing Assay

Transduced HEp-2 and LSCC cells were seeded in 24-well plates and grown to 90% confluence in complete medium. The artificial wound was prepared by scraping the confluent cell monolayer with a 10-µl pipette tip and then washing with PBS to remove the isolated cells. The cells were grown in serum-free medium at 37 °C with 5% CO_2 for 72h, and the wound area was photographed by a microscope (EVOS® FL Cell Imaging System) (Life Technologies) at each time point (0, 24, 48 and 72 hours). Cell migration was assessed and analysed objectively with ImageJ. The wound closure percentage was calculated using the following formula: 1−[24-48 or 72-hour area/0−hour area]. Three independent assays were photographed and quantified.

6.11 *in silico* Targets Prediction

The publicly available algorithms (TargetScan 7.1, DIANA-microT-CDS 5.0, miRANDA-mirSVR e miRmap) allowed to perform an *in silico* analysis of candidate targets of the selected miR-223-3P, moreover we used DIANA-miRPath v.3 web-server for miRNA pathways enrichment analysis. DIANA-miRPath server is able to annotate all the predicted and experimentally validated miRNA targets in a selected molecular pathway, providing accurate statistics, while being able to accommodate advanced pipelines. In fact, miRPath can utilize predicted miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm and/or experimentally validated miRNA interactions derived from DIANA-TarBase v6.0; combine results with merging and meta-analysis algorithms; perform hierarchical clustering of miRNAs and pathways based on their interaction levels; as well as elaborate sophisticated visualizations, such as dendrograms or miRNA versus pathway heatmaps.

6.12 Statistical Analysis

Construction of clustered heatmap for microarray analysis was performed using heatmap.2 function of gplots package in statistical analysis tool R (version 3.4.3). To evaluate expression difference between two groups, student's t-test was used for calculating *p*-value.

Graphs were obtained using GraphPad Prism (version 7.00) and significant differences were determined at $p \leq 0.05$ according to Student's t test.

In receiver operating characteristic (ROC) curve analysis, area under the curve (AUC) was calculated to determine cut off and further calculate sensitivity, specificity and accuracy for investigation of diagnostic performance. To examine relation between miRNA expression levels and interest clinical characteristics, correlation coefficient and *p*-value were calculated by GraphPad Prism (version 7.00).

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***** Gli autori hanno contribuito equamente al lavoro

CONGRESS ABSTRACTS

- **miR-125b upregulates miR-34a and sequentially activates stress adaption and cell death mechanisms in multiple myeloma.** Therapeutic nanoproducts: from biology to innovative technology (Roma, 19-20 Giugno 2019).
- **Nanotech revolution for miRNA delivery in Glioblastoma: a new strategy to overcome temozolomide resistance by targeting O6-methylguanine methyl transferase (MGMT).** Therapeutic nanoproducts: from biology to innovative technology (Roma, 19-20 Giugno 2019).
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- **Comparative study from NGS platform Ion Torrent Personal Genome Machine and Terascreen Rotor Gene Q for the detection of target genes in Lung and Colorectal Cancer and Melanoma.** 1st International and 32nd Annual Conference of AICC "From single gene analysis to single cell profiling: a new era for genomic medicine" (Catanzaro, 1-2 Ottobre 2019)
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- **Definition of a possible miRNA signature for predicting tumor grade and metastasis in Laryngeal Cancer.** 33rd Annual Conference: International Meeting on Cancer Metabolism (Torino, 22-24 Novembre 2021)
- **Dissecting miRNA expression profile modulation throughout preclinical assessment of Ruthenium-based Nanosystems in Triple Negative Brast Cancer.** 33rd Annual Conference: International Meeting on Cancer Metabolism (Torino, 22-24 Novembre 2021)*

*Best Poster Award