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BIRUNI UNIVERSITY

HEALTH SCIENCES INSTITUTE

IN VITRO CHARACTERIZATION of MICRORNAS SPECIFIC TO CANCER STEM CELL IN LARYNX CANCER

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DEPARTMENT OF MOLECULAR BIOLOGY AND GENETIC

MASTER of SCIENCE

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KANSER KÖK HÜCRELERİNE ÖZGÜ MİKRORNA'LARIN LARENKS KANSER HÜCRELERINDE *IN VITRO* KARAKTERIZASYONU

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BIRUNI UNIVERSITY
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SUPERVISOR

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ONAY SAYFASI

Biruni Üniversitesi Sağlık Bilimleri Enstitüsü Moleküler Biyoloji ve Genetik Anabilim Dalında Nur Deniz DİBEK tarafından hazırlanan "In Vitro Characterization of Micro RNAS Spesific to cancer stem cell in larynx cancer " adlı tez çalışması aşağıdaki jüri tarafından YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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Doç. Dr. Leman ŞENTURAN
Sağlık Bilimleri Enstitü Müdürü

DEDICATION

Dear mother, father and brother
My friends who encourage and support me,
All the people in my life who touch my heart...

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I would like to thank my thesis superadvisor Asst. Prof. Elif Sibel Aslan for her invaluable support and supervision during my Master studies.

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And always present to my dear mother, my father, and my brother, who have always supported me with my endless gratitude.

Nur Deniz DIBEK

Biruni Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu

26.02.2018

Sayın: Yrd.Doç.Dr.Elif Sibel ASLAN

Biruni Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu yapılan inceleme sonucunda planladığı **''In Vitro Characterization Of Microrna Specific To Cancer Stem Cell In Larynx Cancer ''** isimli araştırmanızın kurulumuzun **06.02.2018** tarihli toplantısında etik yönden uygun olduğuna karar verilmiştir.

Etik Kurul Başkanı
Prof.Dr.Can Polat EYİGÜN

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LIST of SYMBOLS AND ABBREVIATIONS

RNA : Ribonücleic Acid

miRNA : Micro Ribonücleic Acid

DNA : Deoxyribonücleic Acid

cDNA : Complementary Deoxyribonücleic Acid

mRNA : Messenger Ribonücleic Acid

RNAi : RNA Interference

RISC : RNA Induced Silencing Complex

dsRBD : Double Stranded RNA-binding Domain

RT: Reverse Trancription

qRT-PZR : Quantitive Reverse Transcription Polimerase Chain Reaction

dH_2O : Distilled Water

CO^2: Carbondioxide

µl: Mikrolitre

dNTP : Deoxyribonucleotide triphosphate

HCL : Hydrochloric Acid

FBS : Fetal Bowine Serum

PSA : Penicillin Streptomycin Amphotericin

LSCC : Larynx Squamous Cell Carsinoma

SCC : Squmous Cell Carsinoma

NSCLC : Non-Small Cell Lung Cancer

ABSTRACT

Dibek ND. *In vitro* Characterization of microRNAs Specific to Cancer Stem Cell in Larynx Cancer. Biruni University, Institute of Health Science, Department of Molecular and Medical Genetic. Istanbul. 2017

Larynx cancer is the most common and most aggressive type of cancer in the head and neck region. However, when appropriate diagnosis and treatment is applied, the treatment of the head and neck region is the best responding tumor which is the most common cancer type in the world. The incidence in men is about 10 times higher than in women. The most important risk factors for the development of cancer are smoking and alcohol use. Squamous cell carcinomas constitute 90-95% of laryngeal carcinomas and early diagnosis helps increase the quality of life significantly, and affects the survival time positivelylike in case of the types.

MicroRNAs (miRNAs) are small RNA molecules that do not encode proteins which are actively involved in the regulation of gene expression and in almost all cellular mechanisms. A good understanding of the working mechanisms of miRNAs has been the focus of recent researches. These researches have showed that microRNAs to play important role in the pathogenesis of many cancers such as Larynx Squamous Cell Carsinoma (LSCC)

In this study, samples were first isolated from microRNAs which was already identified in previous microarray studies with mir-26b, mir200c-3p, mir-203, mir363-3p, and mir-1825 in Trizol. cDNA synthesis was followed by qRT-PCR assays. For the microRNAs ready for transfection, Hep-2 cells, the larynx squamos cell line, were used for functional analysis.

After transfection of microRNAs into Hep-2 cells, proliferation, migration and invasion, soft agar analyzes were followed and performed consecutively. Initially, 5 microRNAs were expressed in terms of significance. However, as a result of these analyzes mir-203 and mir363-3p played an important role in these experiments.

As a result of that mir-203 and mir363-3p act as tumor suppressor on Hep-2 laryngeal cancer cells, indicating that cancer cells seriously reduce their ability to proliferate, migrate and invade and colonize. It was determined that these miRNAs could be used during diagnosis. Further experiments and works need to be donefor better understanding of this cancer mechanism.

Keywords: Larynx Cancer , microRNA, mir-203, mir363-3p, Hep-2

Supervisor : Asst. Prof. Elif Sibel ASLAN

OZET

Dibek ND. Kanser Kök Hücrelerine Özgü mikroRNA'ların Larenks Kanser Hücrelerinde *in vitro* Karakterizasyonu, Biruni Üniversitesi, Sağlık Bilimleri Enstitüsü, Moleküler ve Tıbbi Genetik ABD. Yüksek Lisans Tezi. İstanbul. 2017

Baş-boyun bölgesinde en sık görülen ve en agresif olan kanser türü larenks kanseridir. Ancak uygun tanı ve tedavi uygulandığında, baş-boyun bölgesinin tedaviye en iyi yanıt veren tümör ve dünyada en sık tanı koyulan kanser türüdür.Erkeklerde görülme sıklığı kadınlara göre yaklaşık 10 kat fazladır.Bu tür kanserin gelişmesinde en önemli risk faktörleri sigara ve alkol kullanımıdır. Skuamoz hücreli karsinomlar, larenks kanserlerinin % 90-95'ini oluşturmaktadır.Tüm kanser türlerinde olduğu gibi, erken tanı yaşam kalitesini önemli ölçüde yükseltilmesine yardımcı olurken, sağkalım süresini olumlu yönde etkilemektedir.

MikroRNA'lar (miRNA'lar) protein kodlamayan küçük,RNA molekülleridir. Gen ifadesinin düzenlenmesinde ve neredeyse hücresel tüm mekanizmalarda aktif olarak rol oynamaktadırlar. MiRNA'ların çalışma mekanizmalarının iyi anlaşılması son zamanlarda yapılan araştırmaların odak noktası olmuştur. miRNA'ların Skuamoz Hücreli Larenks Kanseri (SHLK) gibi birçok kanserin patogenezinde önemli rol oynadığı gösterilmiştir.

Bu çalışmada, ilk olarak önceki mikroarray çalışmalarında belirlenmiş olan mikroRNA'lar arasından mir-26b, mir200c-3p, mir-203, mir363-3p, and mir-1825 'ın Trizol ile izolasyonları yapılmış ve cDNA sentezi ve ardından qRT-PCR esseyleri yapılmıştır. Transfeksiyona hazır hale gelen mikroRNA'lar için fonksiyonel analizlerde kullanılmak üzere larenks squamos hücre hattı olan Hep-2 hücreleri kullanılmıştır.

Hep-2 hücrelerine mikroRNA'ların transfeksiyonu yapıldıktan sonra sırasıyla, proliferasyon, migrasyon ve invasyon, soft agar analizleri yapılmıştır. Başlangıçta anlamlılık ifade etmesi açısından 5 mikroRNA ile yola çıkılmış ancak yapılan analizlerin sonucunda, bunlardan gözle görülür şekilde mir-203 ve mir363-3p 'nin önemli rol oynadığı bulunmuştur.

Sonuç olarak, mir-203 ve mir363-3p 'nin Hep-2 larenks kanseri hücreleri üzerinde tümor supresör özellikte davranarak, kanser hücrelerinin proliferasyon, migrasyon ve invasyon ve koloni oluşturma yeteneklerini ciddi oranda azalttığı belirlenmiştir. Tanı aşamasında bu miRNA'lardan yararlanabilineceği belirlenmiştir. Bundan sonra yapılacak diğer fonksiyonel çalışmalara da yardımcı olacağı düşünülmektedir.

Anahtar Kelimeler: Larenks Kanseri, mikroRNA, miR-203, miR-363-3p, Hep-2

Tez Danışmanı: Yrd. Doç. Dr. Elif Sibel ASLAN

1.INTRODUCTION

Cancer is a complex disease and multi-step process with epigenetic and genetic changes. Changes in this genetic and epigenetic level cause errors and lead to uncontrolled division of cells. As a result, cancer begins at the cellular level (T.C. Sağlık Bakanlığı Halk Sağlığı Genel Müdürlüğü,2013; Koutsogiannouli*et al*., 2013). There are more than 100 different types of cancer, depending on the organ they are in and the group of cells they are descended from (Sawyers*et al.*,2013). The elaboration of the human genome project and the development of new generation disposal technology and microarray technologies have played an important role in the elucidation of cancer development and development mechanisms in the 2000s (Arnedos*et al.*, 2014).

A better understanding of the molecular mechanism of cancer and the development of studies for diagnosis and treatment are very important. Investigations on cancer-related deaths have been found to be economically disastrous. These economic losses are very important at the country level due to maintenance costs, reduced productivity and premature mortality (Feachem *et al*., 2002; Coleman, 2014). Since 1975, cancer incidence rates have increased for many reasons. However, all investigations for cancer diagnosis and treatment have resulted decline in mortality. The identification of the molecular pathology of cancer is an important step in increasing the quality of life and reducing mortality to a greater extent (Reidenberg and Laitman, 2006).

Approximately 2% of human malignancies are laryngeal squamous cell carcinoma (LSCC). The incidence of LSCC is 7-8 times higher in men than in women. The black race is 1.5-2 times higher than the white race. The incidence of LSCC declines over the past 30-35 years. However, the decline in mortality rates is not enough (SEER Cancer Statistics Factsheets, 2011). The 5-year survival rate was found to be around 60%. LSCC is the 7th most frequent type of cancer in Turkey. The 5-year survival rate for LSCC in our country is below the world average (Türkiye Kanser İstatistikleri, 2014). According to Ministry of Health data, it was determined as 42.8% in 2011. Considering the demographic factors in Turkey, a significant increase in the estimated number of new cases of LSCC between 2015-2030 is

foreseen. Especially in men with LSCC, the mortality rate is expected to increase (SEER Cancer Statistics Factsheets, 2011).

MicroRNAs (miRNAs) are synthesized endogenously as a single chain. They are about 18-24 nucleotides in length and do not encode short ribonucleic acids (RNA). MicroRNAs have recently been one of the most recent cancer research topics (Sevli*et al.,* 2010). Mirna's have been the subject of many studies. The mechanisms of formation, the investigation of the roles in biological processes, the objectives of miRNAs are a few of these studies (Iorio and Croce, 2012). There are also many studies showing that they are actively involved in the pathogenesis of many diseases. In particular, the mechanism of cancer formation has been shown to be deregulation of miRNAs and tumor suppressor characterization oncogenes (Reya*et al*., 2001). The expression of miRNAs with different expression patterns for each different type of cancer has come to the fore in terms of the development of diagnostic and therapeutic methods (Di Leva and Croce, 2013). In recent years, a number of studies have been conducted on the role of miRNAs in carcinogenesis. However, the roles of miRNAs in the carcinogenesis process have not been fully elucidated. Therefore, further studies are needed (Reya*et al*., 2001).

Cancer stem cells are rare cell populations present in the mass of cancer and are thought to be responsible for tumor growth and metastasis by acting like stem cells (Reya*et al*., 2001). Tumor-initiating cells (cancer progenitor cells) have the ability to initiate cancer stem cell tumors and resist resistance to improved therapies (Masters*et al*., 2008). A standard chemotherapy kills most cells in the tumor but does not affect cancer stem cells. Although they are very few, they can lead to tumor recurrence even years after successful treatment. Recent studies have demonstrated the presence of cancer stem cells in many types of cancer, including head and neck, brain, lung, breast, prostate and ovary (Ozen, 2011; Suer *et al.*, 2014). Many studies have shown that miRNAs also play important roles in cancer stem cell regulation. In order to develop miRNA-based therapeutic approaches, it is necessary to clarify the roles of miRNAs in the process of acquiring and maintaining cancer stem cell characterization. there is a need to identify miRNA profiles of cancer stem cells.

As far as the role of miRNAs in laryngeal cancer is concerned, few studies are available. there are studies in which the miRNA expression profile is removed in laryngeal cancer. The effects

of the presence of malignant and benign metaplasia on miRNA (s) in cancer cell proliferation with different expression patterns have also been investigated. The gene through which these effects are carried out has also been identified and reported (Zhao*et al*., 2013; Xu*et al*., 2013). A limited number of studies on laryngeal cancer related to cancer stem cells have also been published. The first study was conducted by Wei et al. They have been published as a study to investigate CD133 as a possible cancer stem cell marker in the Hep-2 cell line. In this study, it was reported that a small group of cells in the Hep-2 cell line were CD133 +, and this cell group had an increased tumorigenesis capacity (Wei*etal*., 2009). In another study, a 'side population' characterization was performed in Hep-2 cells. In these cells, increased self-renewal, proliferation, radiotherapy resistance and tumorigenicity have been demonstrated (Wan*et al*., 2014).

A comprehensive study of the effects of cancer stem cell-specific miRNAs on laryngeal cancer cells and laryngeal cancer stem cells has not yet been conducted as far as we know. The aim of this study is to investigate the effects of miRNAs on cancer cells with oncogenic or tumor suppressor effects in cancer stem cells from squamous cell laryngeal carcinoma. In this study, human laryngeal squamous cancer cell line Hep-2 cells were treated with miR-26b, miR-200c-3p, miR-203, miR-363-3p and miR-1825 mimics to determine tumor cell proliferation, migration and invasion, and functional effects on stem cells.

1.1.Larynx Anatomy

The larynx is an organ which segment of 3-6 neck vertebrae in front of the neck. It has responsible of respiration and conversation functions (26).

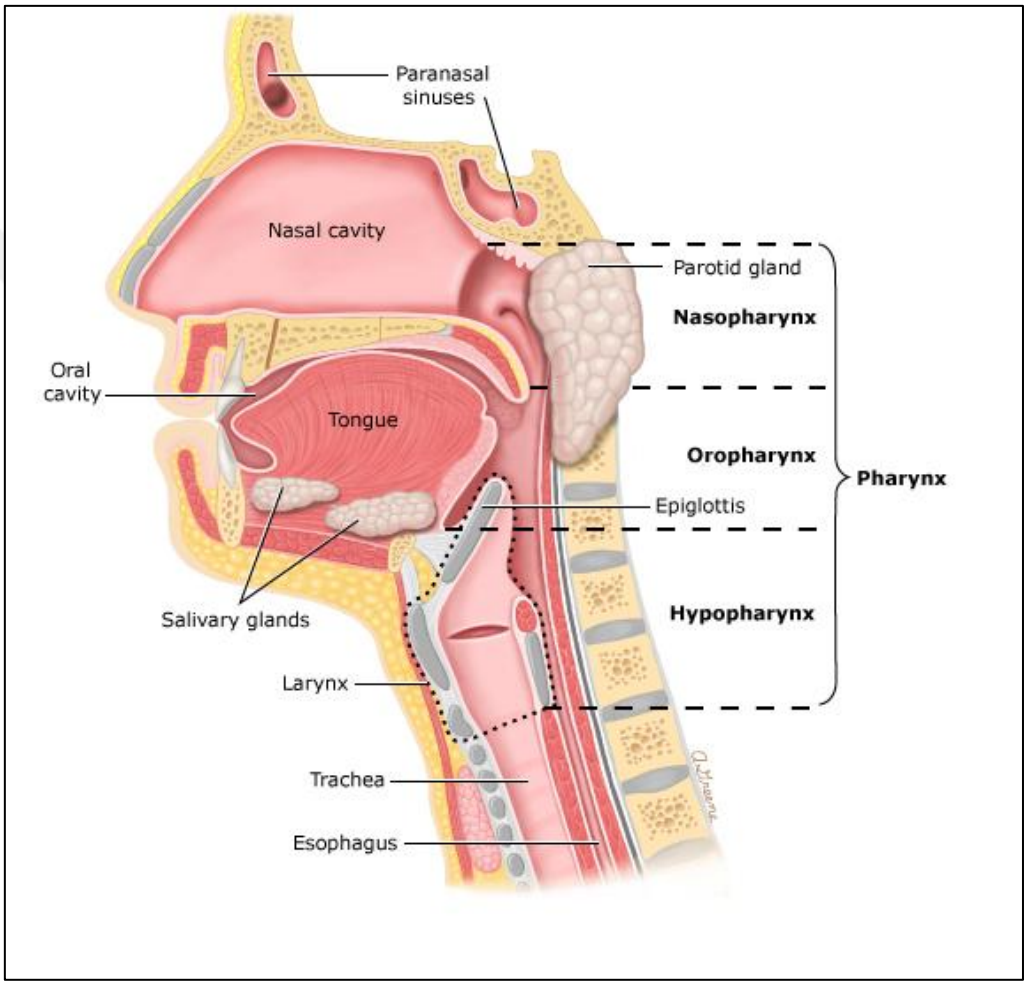


Figure 1.1: An illustration showing possible cancer sites in different regions of the head and neck. It is adapted from (Brockstein*et al.*, 2016)

The larynx is localized between the tongue and trechea. The trachea is in interaction with the lower part of the pharynx.And the trachea creates the front wall of the pharynx (Odar, 1980). It is beginned from epiglottis and it is continued the cricoid cartilaginous as anatomic.The total cartilages are 9 including 3 singles and 3 doubles. Aritenoid, kuneiform, and corniculat are pair cartilages. 3 singles cartilages are the thyroid, cricoid, epiglottis (Kaya, 2002).

The upper zone of the larynx canconstrict the respiratory tract and close completely when needed. Foreign substances is prevented the larynx muscles from passing to the respiratory tract.In addition, the larynx muscles also played a role in altering the shape of the laryngeal cartilage. It had an actions on the voice coil also (Odar, 1980).

The larynx has the ability to regulate air flow during exhalation. It also has many important vital functions related to functions such as speaking, swallowing and breathing (PL, 2007).

The protecting the lower respiratory tract is the most important function of the larynx. During swallowing, the larynx closes the parenchyma and temporarily stops breathing. And the food marches to the respiratory tract. In addition, Laryngeal foreign substances also provide a cough reflex that occurs when they interact with respiratory tract mucosa (PL, 2007).

Other tasks of the larynx; the formation of the speech function, the regulation of the breathing process, balancing the rib cage (PL, 2007).

Fixation of the thoracic cage is a necessary condition for optimally operation of numerous thoracic and abdominal muscles. Closing the glottis and fixing the rib cage, coughing, vomiting, diarrhea and urination, and giving birth are performed (PL, 2007).

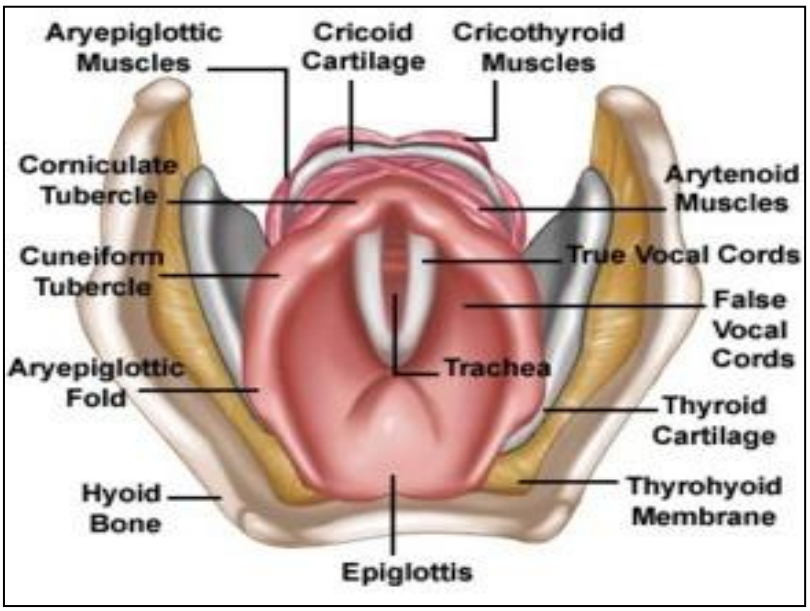


Figure 1.1 :Laryngeal anatomy in the coronal plane from a anterior view.

Picture is adapted from (143)

1.2.Embryology of Larynx

In the 4th week of the embryological development occurs with the deeping of the slice. This formation begins from the ventral wall of the pharynx in the middle laryngotracheal clef. This is the first indicator of the future lower respiratory system in the 4mm embryo. This tube is covered with endoderm. The head of the laryngotracheal tube creates the larynx and trachea. The lower end comprises two lateral protuberances.And then, bronchus, right and left lung lobes develop (Pansky, 1982; Weir, 1997).

The larynx is of epidemic endoderm origin and cartilage, muscles are of mesenchymal origin. The increasing of interaction formed an opening T shape in the laryngeal space. The laryngeal cavity begins to gain its typical structure when the cecum turns into thyroid, cricoid, and arytenoid cartilages (Kozuma *et al.,* 1991).

Laryngeal lumen obstruct due to rapid proliferation of the laryngeal epithelium as temporarly. Until the tenth week, the larynx re-canals and laryngeal ventricles form. These ventricles are surrounded by tissue cathelons that will later change to pseudo-real vocal cords (Kozuma *et al.,* 1991). The larynx which continues to develop during the postnatal period is completed around the age of 65 (Cummings, 1998).

1.3.Histology of Larynx

According to studies performed in the newborn, the larynx initially had silical epithelium in the areas outside the vocal cord. In the sixth month the pseudo-vocal cord was squamous epithelium. The stratum squamous epithelium was laid on the tongue or anterior of the epiglottis. The epiglottis lingual face is covered by multilamellar epithelium, and the upper part of the laryngeal (back) side changes from multilamellar to flattened epithelial to columnar columnar epithelium. The transition between these two types of epithelium consists of a sharp or 1-2 mm transitional zone. The supraglottic and infraglottic part of the larynx is covered with the serrated columnar epithelium while the glottic part is covered with the multilayered flat epithelium (Koç, 2003).

The multi layered flat epithelium, composed of many cell layers, allows the tissue to be very thick. Cells on the surface can be cubic or prismatic, with cells on the basal lamina that are flat and responsible for cell production. As new cells grow, older cells push up and these

cells flatten. In the body mouth, esophagus, a part of you epiglotti the cornea and the epidermis of the skin in various areas such as in the cells of the multi layered flat epithelium can be found (Wikipedia, 2014).

Half of non-smoking adults have squamous epithelium areas in the ciliated repiratory type epithelial areas, supra- and infraglottic areas. In smokers, the respiratory epithelium of the larynx can completely replace the squamous epithelium (Juan, 2004; ,O, 2002; Metzger*et al*., 2011).

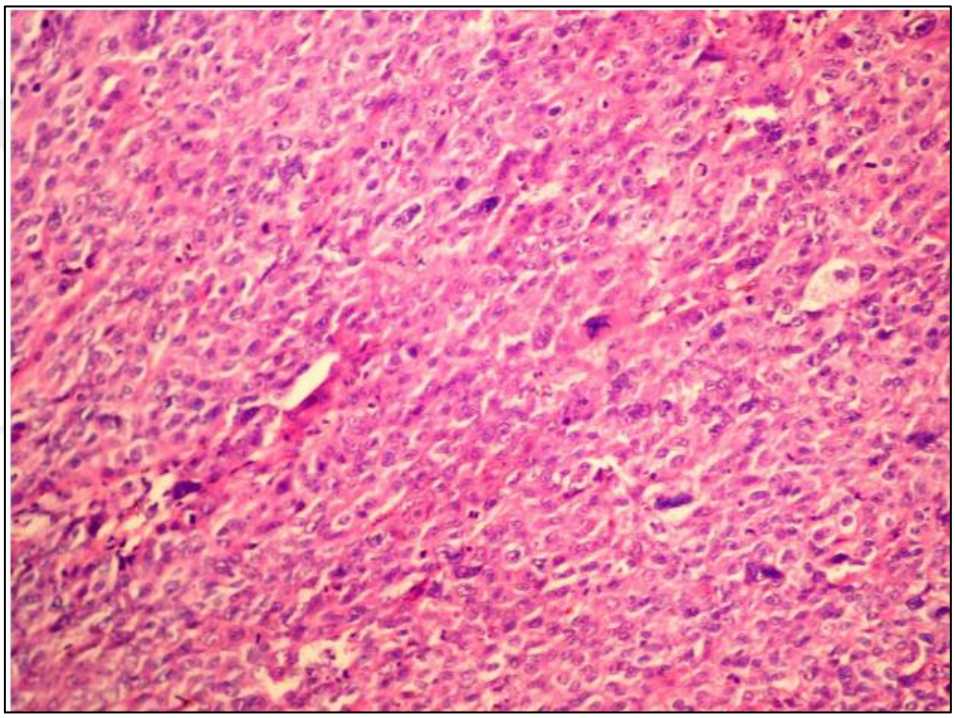


Figure1.3: The histology of larynx squamose cell carcinom is shown in the figure. Picture is adapted from (Sayılgan, 2006).

1.4.Physiology od Larynx

Larynx has three basic functions (Som and Curtin, 2003).

a-Lower respiratory tract protection function

The larynx closes the respiratory tract when swallowing is performed after ingestion of food and prevents the ingestion of liquid nutrients to the lungs (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2017).

b-Breathing Function

While breathing, the Sekitali and vocal tracts open naturally to allow air to enter and exit the lungs via the trachea. If something other than air enters the trachea, a cough reflex is triggered (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2017).

c-Speaking Function

To speak, the larynx produces sound. Sound is produced when the airborne sound vibrates. the audio cables move to change the volume and tone of the sound. By moving your mouths, your language and your lips from the air through the air larynx, you will create different voices (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2017).

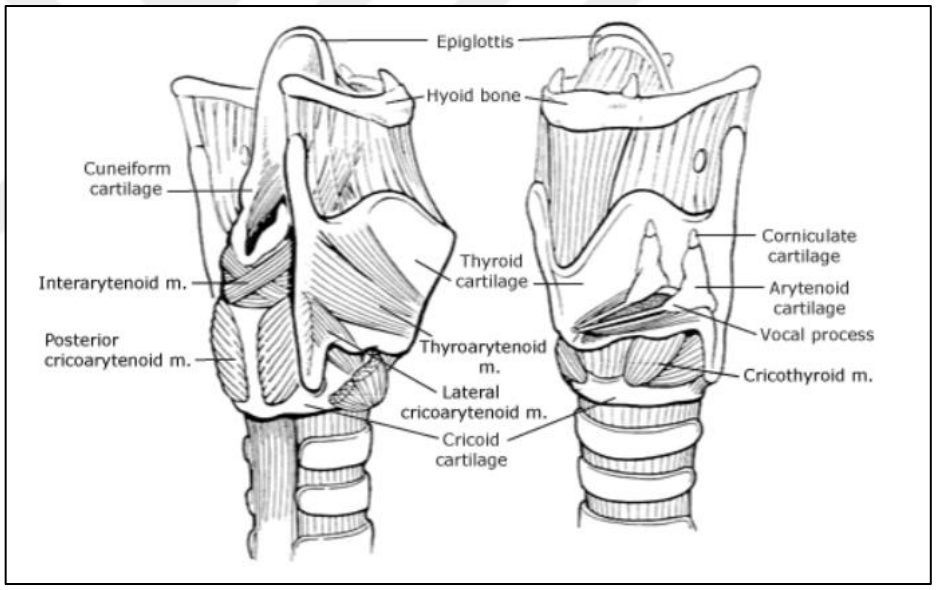


Figure 1.4 : It is shown the speech production of larynx. Picture is adapted from (Brunch et al., 2017).

1.5. Larynx Cancer

Cancer is a disease that occurs with highly variable genetic and epigenetic changes. Cancer is more common in developing countries than in developed countries, according to the cancer assessment in the world (Figure2-3).It is significant to understand the complexity and polymorphisms of cancer in functional studies. The onset and development of cancer in

humans is characterized by various mutations, chromosomal disorders, and increased / decreased gene expressions. Increased transcript levels in cancerous genomes are associated with increased number of gene copies with the proliferation of oncogenes and inactivation of tumor suppressor genes (Oktem *et al*., 2000).

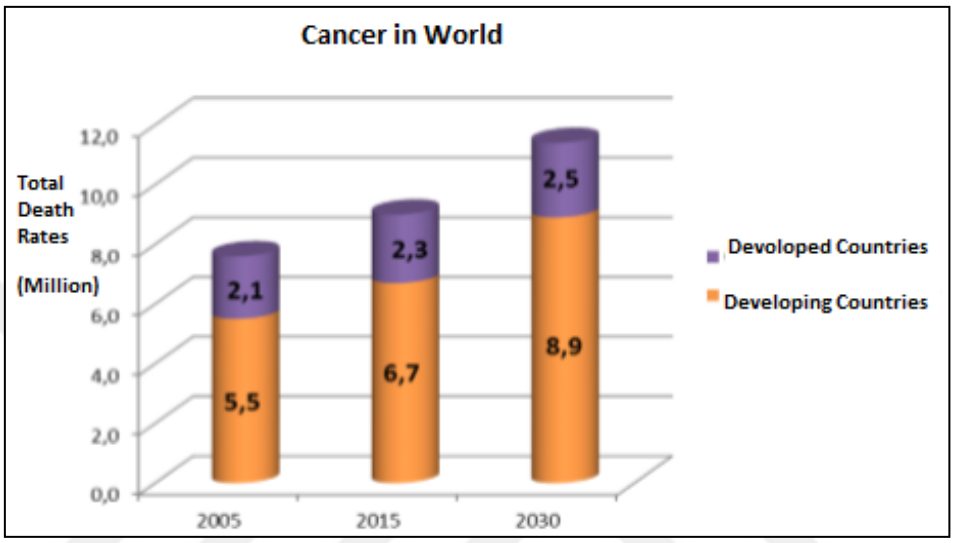


Figure 1.2 : The Rates of Death in Developed and Developing Countries. It is adapted from (T.C. Sağlık Bakanlığı Türkiye Halk Sağlığı Kurumu,2014).

According to investigator in 2012, all cancer event determined as about 14.1million in worlwide. This state shown 7.4million in men. The 6.7 million case is shown in womens. By 2035, this number is estimated to rises as 24 million (Ferlay*et al*.,2015). Cancer is the leading health problem in the world. United state te is also the second health problem. Cancer case occured 1,685,210 and cancer deaths is 595,690 in United State in 2016 (Rebecca*et al.,* 2016).

Head and neck cancers represent various epithelial tumors that occur in the oral cavity, pharynx, larynx, and nasal cavity. This type of cancer is the sixth most common cancer worldwide, with approximately half a million new cases each year.These cancers, whose incidence is still increasing in various geographical regions, are also affecting young people nowadays (Leemans*et al*., 2011).

Laryngeal cancer is seem accounts for 2% of all cancers in adults and 2.1% of all cancer deaths worldwide. It is the second most common cancer of head and neck squamous cell carcinomas. Laryngeal cancer is also the most common type of cancer among upper

respiratory tract cancers. And 90-95% are composed of squamous (flat) cell carcinoma (SCC) or epidermoid carcinoma variations. In addition, 25% of these tumors in the head and neck region are located in the larynx (Oktem*et al*., 2000;Kirchner and Carter, 2004;Moore*et al*., 1999; Farrag*et al*., 2006).

The most frequent age of laryngeal carcinoma is reported to be 1% in the younger ages, with a concentration in the 50-70 (Kirchner and Carter, 2004;Mao*et al.,* 2012;Zhang*et al.,* 2013). It have been reported in younger age groups also. The incidence of laryngeal cancer varies according to the countries. In general, the incidence is in the range of 3-10 / 100.000. (Gallus et al.,2003). The highest incidence for 2012 data was found in Cuba with 7.6 / 100.000 per year. Other common countries are Georgia, Armenia and Hungary with an incidence of 5.7-6.3 / 100.000. The ranking is followed by Belarus, France, Turkey and Spain with an incidence of 3.8-4.1 / 100.000. According to the statistics, it is found that in countries like Africa, East Asia, New Zealand and Norway, it is below 1.9 / 100.000 (Globocan, 2014).

The ratio of male / female with larynx cancer reported as 5: 1 in many countries is 30: 1 in some countries (Gallus*et al.,* 2003;Topuz*et al*., 2000). Although laryngeal cancers are reported as 5: 1 in many countries, the male / female ratio is 30: 1 in some countries (Gallus*et al.,* 2003;Topuz*et al*., 2000). This ratio has been shown in some sources around the world as 7: 1. (Mirisola*et al.*,2011). When the Ministry of Health records in Table 2-1 are evaluated, larynx cancer is the 7th most common cancer in Turkey according to the year 2011 data. In patients with laryngeal cancer, 4-30% of secondary cancers can develop, including lung, head and esophagus (Gao*et al.,* 2003).

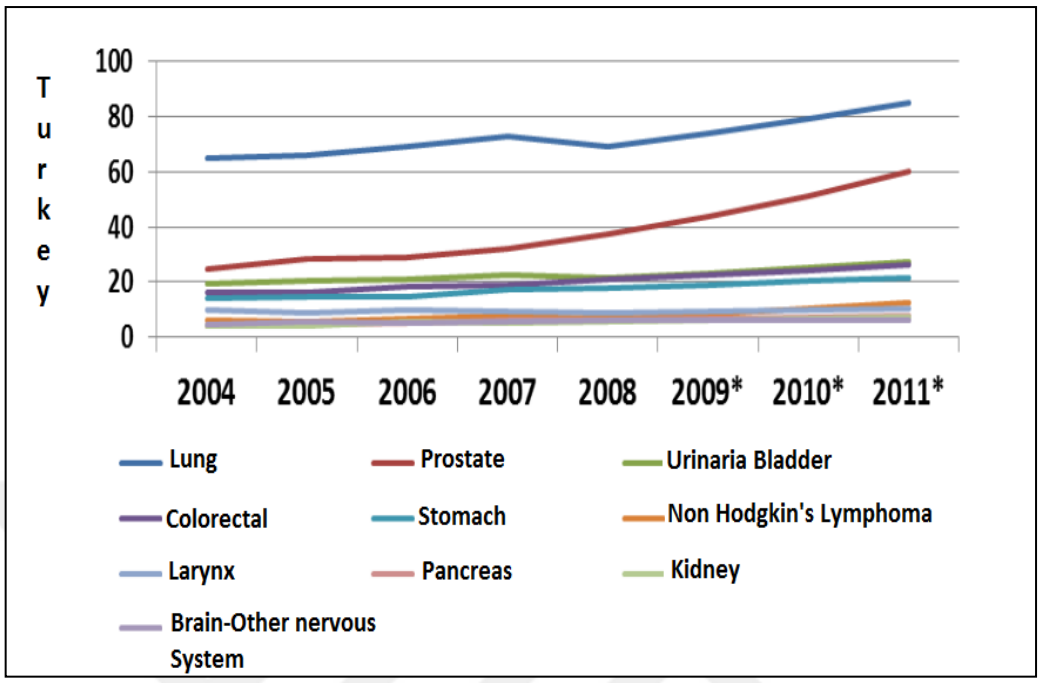


Figure 1.3 : The incidence of the 10 most common cancer types in men (the incidence at 100,000). It is modified from (Sağlık İstatistikleri Yıllığı, 2014).

1.5.1.Larynx Cancer Etiology

Larynx cancer is happened a combination of genetic factors and factors in your environment. Tobacco use is one of the major factors that increase the risk of developing laryngeal cancer. Alcohol use is also associated with the risc of developing cancer of the larynx (Head and Neck Cancer Guide, 2017; Epocrates, 2017).

In the etiology of laryngeal cancer, there are also many factors such as chemicals such as paint-gasoline, wood dust, asbestos, various metal powders, hormonal state of the person, dietary diet, sulfuric acid, coal and other factors that can lead to air pollution and radiation.

According to the research, 95% of the patients with laryngeal cancer were found to have cigarette consumption. Duration of smoking and the number of annual packs were found to affect the aggressiveness of laryngeal cancer. At the same time, 10-15 years after leaving the smoking cessation, the risk factors can go away. On the contrary, it has been argued that the effect of smoking cessation is small because of the cumulative damages in the cell in some studies (Koufman and Burke, 1997; Tomek and McGuirt, 2003).

Smoking and alcohol users, according to those who do not use larynx cancer appeared about 10 years early.In those who do not smoke or drink alcohol, these cancers are frequently located in the glottic region with low metastatic capacity. In addition, the survival rates of these people were higher (Agudelo*et al*., 1997).

Alcohol after smoking is also an important risk factor that plays a role in laryngeal cancer (Oktem, 2000;Demireller, 2004). Alcohol and cigarettes are found to be synergistically carcinogenic when used together (Bosetti *et al*., 2002). According to one study, the risk of laryngeal cancer was 2.46-times higher in non-smoking alcoholics and 9.38-times in smoking-free non-alcoholics. Those who consume both alcohol and cigarettes has been found to increase the risk 26 times (Koufman and Burke, 1997).

In recent years, viral factors (Epstein Barr Virus, Human Papilloma Virus) may play a role in the etiology of squamous cell carcinomas of the larynx. (Manjarrez*et al*., 2006; Engin, 2003). Not definitively proven, gene polymorphisms are other risk factors. Especially due to the damage caused by the carcinogenic substances contained in the cigarette, attention is paid to the polymorphisms of the genes involved in DNA repair (Busquets*et al.,* 2003). The relationship between cyclin D1 gene amplification and glottic cancers is emphasized in Monteiro *et al.* (Monteiro*et al.,*2004). As with other types of cancer, it is reported that the hormonal status of the person and the imbalances in the diet are also risk factors for laryngeal cancer.(Riboli*et al.,*1996)

1.5.2.Larynx Cancer Pathology

Many types of benign and malignant tumors can be seen in the larynx.Non-Neoplastic tumors such as vocal nodules, vocal polyps, contact ulcers, ductal and sacrocular ulcers, neoplastic tumors such as laryngosclera, chondroma,squamous papilloma, granular cell tumor and glandular tumors are benign tumors of the larynx (PL, 2007).

Squamous cell carcinoma (SCC, squamous cell carcinoma) is the most common site melanoma. The second most frequently observed region is the larynx (Kaya, 2002). Squamous cell carcinomas contains 90-95% of larynx cancers (Epocrates, 2017).

Besides, these carcinomas is seem in larynx :

- Small cell carcinoma
- Mucoepidermoid carcinoma
- Adenocarcinoma
- Adenoid cystic carcinoma
- Sarcomas (chondrosarcoma, fibrosarcoma, rabdosarcoma, etc.)
- Epidermoid carcinoma variations (Kaya, 2002)

Supraglottic cancers: The supraglottic region is rich in lymphatic. For this reason, these tumors are at an increased risk of lymphatic metastases because of the location of the epiglottis from the free end to the ventricle (Myers and Alvi, 1996). The prognosis of these cancers is worse due to they can easily locate the thyroid cartilage and spread to the preepiglottic area (Rosai, 2004). Supraglottic cancers constitute about one-third of Larynx cancers and are more aggressive than glottic cancers (Koch *et al*., 2017).

Glottic cancers: These cancers usually develop as a good differential tumor(grade1). They can usually spread vocal by spreading forward and backward. The risk of lymphatic metastasis is low because the glottic region is quite poor from lymphatic.The prognosis is better. However, prognosis worsens if placement of the anterior commissures is concerned (Engin and Erişen, 2003). About two-thirds of the larynx cancers constitute glottic cancers (Koch *et al*., 2017).

Subglottic cancers: These tumors have a high metastatic capacity. They can extend from the vocal chord 1 cm below to the lower edge of the cricoid cartilage.They frequently metastasize the Delphian lymph node, and the rate of spread of cricoid and thyroid cartilage is high.Metastases can localize in the glottic and supraglottic regions, esophagus or thyroid, under the trachea and cricoid cartilage (Janfaza*et al.,* 2002). This type is an uncommon type of laryngeal cancer (Koch *et al*., 2017).

Transglottic cancers: The glottis is a supraglottic or subglottic tumor that passes through the vertical (vertical) and carries another region. Cervical lymph node metastasis is high.They can be spread to the paraglottic area in the early period. Thus, prognoses of this cancers are worse than other types (Rosai, 2004; Engin and Erişen,2003).

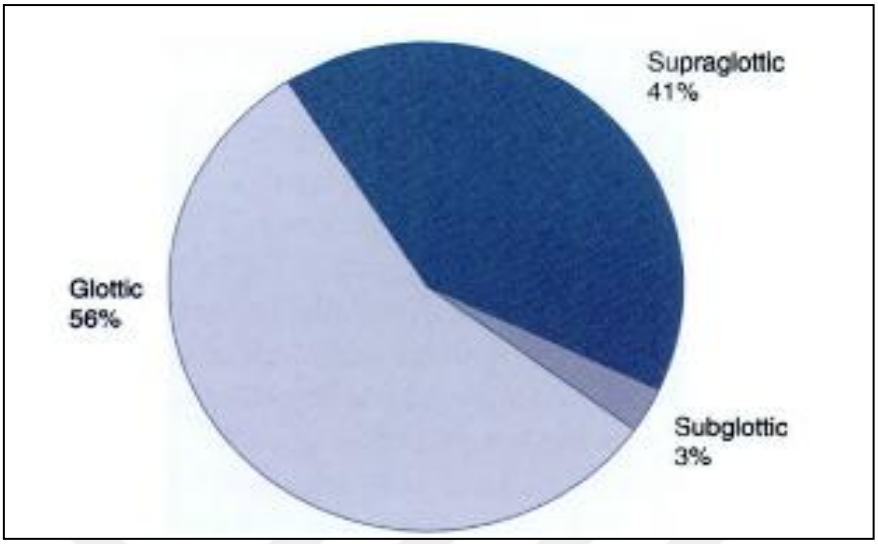


Figure 1.4 : The incidence of the 10 most common cancer types in men (the incidence at 100,000). It is modified from (Lore, 2005**).**

Laryngeal carcinomas are divided into 3 types according to dif+ferentiation grade and mitotic activities: good differentiation (Histologic grade 1), moderate differentiation (Histologic grade 2) and less differentiation (undisturbed) (Histologic grade 3) (Rosai,2004; Kaya, 2008)

According to studies performed, less differential tumors are associated with poor prognosis. There was no statistical difference in the prognosis between good and moderately differentiated tumors (Yilmaz*et al*., 1999). Also histological grade is effective on radiotherapy activity. The better regional control was seen in grade 1 and grade 3 tumors (Fortin*et al*.,2001). Besides, it is stated that there is less risk of early metastases in grade 1 cancers. It is stated that it is possible to cure by surgical treatment in general (Kaya, 2008).

1.5.3.TNM Staging System

With the right treatment options chosen, patients with laryngeal cancer will benefit from the TNM stage, which is preferred all over the World. It was originally developed by Denoix in 1940. The staging system is currently regulated by two organizations that continue with revisions (AJCC Cancer Staging Manual, 1997). American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) is used to classify cancers.

The T classifications indicate the extent of the primary tumor and are site specific; there is considerable overlap in the cervical node (N) classifications (Poon and Stenson, 2017).

The TNM system does not contain the clinical and biological properties of cancer. ' T 'tumor, metastasis to the' N 'regional lymph nodes, and' M 'to the distal metastasis. The locus of the tumor and the vocal cord mobility give the T phase. Lymph node size and size are used for N staging. M is distant metastases, except neck and larynx (Curran*et al.*, 1999).

Primary tumor (T)	
Supraglottis	
T category	**T criteria**
TX	Primary tumor cannot be assessed
Tis	Carcinoma *in situ*
T1	Tumor limited to one subsite of supraglottis with normal vocal cord mobility
T2	Tumor invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis (eg, mucosa of base of tongue, vallecula, medial wall of pyriform sinus) without fixation of the larynx
T3	Tumor limited to larynx with vocal cord fixation and/or invades any of the following: Postcricoid area, preepiglottic space, paraglottic space, and/or inner cortex of thyroid cartilage
T4	Moderately advanced or very advanced
T4a	Moderately advanced local disease. Tumor invades through the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx (eg, trachea, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus).
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Glottis	
T category	**T criteria**
TX	Primary tumor cannot be assessed
Tis	Carcinoma *in situ*
T1	Tumor limited to the vocal cord(s) (may involve anterior or posterior commissure) with normal mobility
T1a	Tumor limited to one vocal cord
T1b	Tumor involves both vocal cords
T2	Tumor extends to supraglottis and/or subglottis, and/or with impaired vocal cord mobility
T3	Tumor limited to the larynx with vocal cord fixation and/or invasion of paraglottic space and/or inner cortex of the thyroid cartilage
T4	Moderately advanced or very advanced

T4a	Moderately advanced local disease. Tumor invades through the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx (eg, trachea, cricoid cartilage, soft tissues of neck including deep extrinsic muscle of the tongue, strap muscles, thyroid, or esophagus).
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Subglottis	
T category	**T criteria**
TX	Primary tumor cannot be assessed
Tis	Carcinoma *in situ*
T1	Tumor limited to the subglottis
T2	Tumor extends to vocal cord(s) with normal or impaired mobility
T3	Tumor limited to larynx with vocal cord fixation and/or invasion of paraglottic space and/or inner cortex of the thyroid cartilage
T4	Moderately advanced or very advanced
T4a	Moderately advanced local disease. Tumor invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx (eg, trachea, soft tissues of neck including deep extrinsic muscles of the tongue, strap muscles, thyroid, or esophagus).
T4b	Very advanced local disease. Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures.
Regional lymph nodes (N)	
Clinical N (cN)	
N category	**N criteria**
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension and ENE(–)
N2	Metastasis in a single ipsilateral node, larger than 3 cm but not larger than 6 cm in greatest dimension and ENE(–); **or**Metastases in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE(–); **or**Metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE(–)
N2a	Metastasis in a single ipsilateral node, larger than 3 cm but not larger than 6 cm in greatest dimension and ENE(–)
N2b	Metastases in multiple ipsilateral nodes, none larger than 6 cm in greatest dimension and ENE(–)
N2c	Metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE(–)
N3	Metastasis in a lymph node, larger than 6 cm in greatest dimension and ENE(–); **or** Metastasis in any lymph node(s) with clinically overt ENE(+)
N3a	Metastasis in a lymph node, larger than 6 cm in greatest dimension and ENE(–)

N3b	Metastasis in any lymph node(s) with clinically overt ENE(+)
NOTE: A designation of "U"or "L"may be used for any N category to indicate metastasis above the lower border of the cricoid (U) or below the lower border of the cricoid (L). Similarly, clinical and pathological ENE should be recorded as ENE(–) or ENE(+).	
Pathological N (pN)	
N category	**N criteria**
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension and ENE(–)
N2	Metastasis in a single ipsilateral lymph node, 1,18 in or smaller in greatest dimension and ENE(+); **or**Metastasis in a single ipsilateral lymph node, larger than 3 cm but not larger than 6 cm in greatest dimension and ENE(–); **or**Metastases in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE(–); **or**Metastasis in bilateral or contralateral lymph node(s), none larger than 6 cm in greatest dimension and ENE(–)
N2a	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension and ENE(+); **or**Metastasis in a single ipsilateral node, larger than 3 cm but not larger than 6 cm in greatest dimension and ENE(–)
N2b	Metastases in multiple ipsilateral nodes, none larger than 6 cm in greatest dimension and ENE(–)
N2c	Metastasis in bilateral or contralateral lymph node(s), none larger than 6 cm in greatest dimension and ENE(–)
N3	Metastasis in a lymph node, larger than 6 cm in greatest dimension and ENE(–); **or** Metastasis in a single ipsilateral node, larger than 3 cm in greatest dimension and ENE(+); **or**Metastases in multiple ipsilateral, contralateral, or bilateral lymph nodes and any with ENE(+); **or**A single contralateral node 3 cm or smaller and ENE(+)
N3a	Metastasis in a lymph node, larger than 6 cm in greatest dimension and ENE(–)
N3b	Metastasis in a single ipsilateral node, larger than 3 cm in greatest dimension and ENE(+); **or**Metastases in multiple ipsilateral, contralateral, or bilateral lymph nodes and any with ENE(+); **or**A single contralateral node 3 cm or smaller and ENE(+)
NOTE: A designation of "U"or "L"may be used for any N category to indicate metastasis above the lower border of the cricoid (U) or below the lower border of the cricoid (L). Similarly, clinical and pathological ENE should be recorded as ENE(–) or ENE(+).	
Distant metastasis (M)	
M category	**M criteria**
M0	No distant metastasis
M1	Distant metastasis
Prognostic stage groups	

When T is...	And N is...	And M is...	Then the stage group is...
Tis	N0	M0	0
T1	N0	M0	I
T2	N0	M0	II
T3	N0	M0	III
T1, T2, T3	N1	M0	III
T4a	N0, N1	M0	IVA
T1, T2, T3, T4a	N2	M0	IVA
Any T	N3	M0	IVB
T4b	Any N	M0	IVB
Any T	Any N	M1	IVC

Table 1.1 : TNM staging of Cancer of the larynx in AJCC UICC 2017. Table is adapted from(Poon et al., 2017)

1.5.4.Diagnosis and Treatment of Larynx Cancer

The most common symptoms of laryngeal cancer; It is muffled voice, change of voice, dry throat, constant cough, pain in the throat swallowing, difficulty in swallowing, earache, difficulty in breathing, weight loss and tuberage in throat region.

Many techniques are used for the diagnosis of laryngeal cancer. These techniques include direct or indirect laryngoscopy, micrographoscopy, magnetic resonance imaging (MRI), biopsy examination, scanning, computed tomography (CT) scanning chest film, positron emission tomography (PET) scanning (Laryngeal and Hypopharyngeal Cancer, 2012).

Most of the laryngeal tumors are localized to the glottic region at about 56%. The sequence follows the supraglottic region (41%) and the least common supraglottic region (1-2%). The identification of the tumor origin is of great importance. Clinically, there is an increased awareness of the preference of the treatment modality for different behaviors and for the knowledge of this difference in later processes (Carew, 2001).

Conventional conservative surgical intervention, endoscopic laser surgery, supracricoid partial or total laryngectomy, radiotherapy and combined chemotherapy are used in LSCC treatment. However, new treatment approaches need to be developed. Because the incidence of LSCC incidence and mortality is not at the desired level and there is no increase in 5-year

survival rates. There is an expectation for an increase in estimated incidence and mortality rates for Turkey. Therefore, clarification of the pathogenesis of LSCC is of great importance in this process (Karataş, 2014).

1.6.microRNAs

MicroRNAs (miRNAs) are a class of non-coding RNA gene which 18-22 lenght. MiRNAs has important role in cellular mechanism and genetic pathogenesis as deregulation (Jones*et al*., 2006). Precursor miRNAs is long RNA which from 100 to 1000 various lenght. Pri-miRNAs are produced by RNA polimerase II enzyme. Pri-miRNAs are translated short miRNA-miRNA duplex, and then they are turned single strand mature miRNAs. Mature miRNAs are binded messenger RNA sequences.Expression of the genes of interest inhibits either transcription either by inhibiting translation or by triggering RNA degradation.It has been described that microRNAs link to mRNAs in this way, affecting 30-90% expression of human genes (Friedman*et al*., 2009).

The chromosomal location of the detected RNAs is linked genetic alterations responsible for the development of some types of cancer. Most miRNAs are located in fragile parts. The fragile parts are regions where the heterozygote disappears. According to the studies performed, these fragile regions are seen where amplification is rarely seen or where chromosomal breakpoint occurs (Wijnhoven*et al.*, 2007). In some human cancer types, in multifactorial disorders and in the genetic pathogenesis, abnormal escape of miRNAs is critical (Biasiolo*et al*., 2011).

It has led to many new cases since the discovery of regulation of transcriptional gene expression of miRNAs. MiRNAs biological processes such as cell differentiation, apoptosis, organogenesis, and the emergence of gentle regulation in the pathogenesis of diseases (Brennecke*et al*., 2003; Chen*et al*., 2004). microRNAs are profiling by various methods such as blotting, RT-PCR and microarray (Croce, 2009).

The frequency and unique structure of miRNA molecules, in addition to being biomarkers and also providing therapeutic uses.Recent studies demonstrate that miRNAs are prognostic markers in clinical aggressive and recurrent cases. The use of MiRNAs both as a new cancer

marker and as a diagnostic / prognostic tool has provided many advantages (Gordanpour*et al.*, 2012).

1.6.1.The Discovery of microRNAs

microRNAs and short interfering RNA(siRNA) is two well defined small RNA types. MiRNA and siRNA is not recognized as biochemical and functional. They is defined according to where came from. MiRNAs occurs precursors of hairpin of dsRNAs. SiRNAs consist of long dsRNAs (Karagün*et al*., 2014).

MicroRNAs are functional RNA molecules. Provide transcription from the RNA genes in the intron or exon regions encoding the protein on the genome and in regions that do not encode the protein. But, protein translations of miRNAs do not materialize in this time miRNAs in the human genome hundreds of high-level encoders encoding the gene region has been discovered. Recently, over 1000 miRNAs has been defined in human genom (Karagün*etal*., 2014; Shenouda and Alahari, 2009).

First miRNAs discovered by Lee *et al.*in 1993 in nematode Caenorhabditis elegans found.Lin-4 gene is important for *C. elegans* in post embriyonic development. Altough this gene does not code a protein, the gene converted 22-nucleotide RNA molecule (Espinosa and Slack, 2006). However, the microRNA term for this RNA molecule has been used for the first time since 2001 (Lee*et al*., 1993; Ruvkun, 2001).

This molecule suppress the expression of lin-4 mRNA with its untranslated region (UTR) as directly. First times, This subject accepted as special feature of *C.elegans*. But, this approved as a new method of gene regulation (Espinosa and Slack, 2006).

miRNA was discovered by Reinhart *et al*. in 2000 in *C.elegans*. Let-7 gene which regulated development timeline of *C.elegans* was found. This small RNAs play the crucial role regulation of gene regulation. This discover followed in the worm, fly and mammalian genomes (Espinosa and Slack, 2006; Reinhart*et al.*, 2000; Pasquinelli*et al*.,2000).

1.6.2.microRNAs Biology

miRNAs are small non-coding RNAs which 18-24 nucleotides in lenght. miRNAs regulates proteins as post transcriptional. miRNAs causes inhibation of protein translation or degredation of messenger RNA(mRNA). According to prediction, more than 60% of

mammalian mRNA are targeted by at least one miRNA. Recent studies shown that miRNAs play critical roles in many cellular mechanism such as replication, regenration, differentation, proliferation, apoptosis (Karagün*et al*., 2014).

miRNAs influence gene expression by regulating gene expression after transcription, and are encoded by genes that do not translate into proteins. miRNAs are transcripts as primiRNAs (primers). These transcripts consist of a sequence of primers and a poly-A tail. Transcripts are transformed into precursor structures called pre-miRNA (precursor) in the cell nucleus. The pre-miRNAs consist of about 70 nucleotides (Kim, 2009). Microprocessor complex miRNAs, which consist of the Pasha (DGCR8) protein groups that link to the crosshairs and double-stranded RNA, provide the software (Denli*et al*., 2004).

Exportin 5 carries the small hairpin pre-miRNA to the placenta by the GAN-GTP mechanism. Pre-miRNAs are not active. It combines with the digested endonuclease and transforms into mature miRNAs in the cytoplasm. This cleavage is catalyzed by the double-stranded RNA binding domain (dsRBD) and Dicer, which simultaneously constitutes the RNAz III enzyme complex with TRBP / PACT (Siomi and Siomi, 2010).

The formation of the RNA-induced silencing complex (RISC) with dicer is initiated. RISC miRNAs and RNA interferon (RNAi) (Bernstein*et al.*, 2001). The digger cuts the strings of the premiers. As a result, two complementary complementary RNA molecules are formed. These are 5 'stable nucleotides. This chain is included in this complex through Argonat, the RNAz function in the RISC complex. This is the yarn guide (Preall*et al*., 2006). The other thread, the substratum of the RISC complex, is called the anti-guide. After the miRNAs bind to the RISC complex, the Argonat proteins provide the degradation of the mRNAs by making base transcription with complementary mRNAs (Gregory*et al*., 2005).

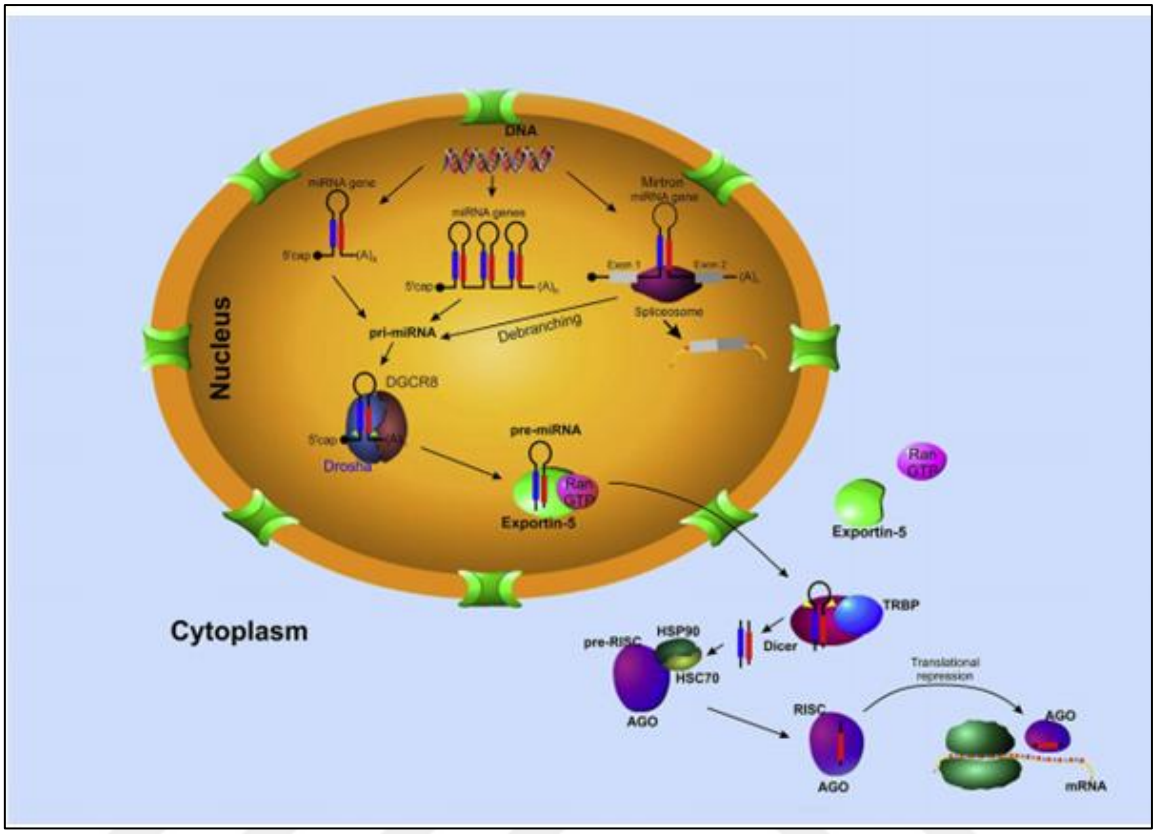


Figure 1.5: The figures showing the miRNA biogenesis. Picture is adapted from (Króliczewskia *et al*., 2018)

Transcription of pri-miRNA transcribe begins by RNA polimerase II. Pri-miRNAs are processed in the nucleus by Drosha and pre-miRNA hairpins occur. Pre-miRNAs tranferred by Exportin-5 in cytoplasm. Hairpin of pre-mRNAs tranformed mature miRNA which 18-24 nucleotides by Dicer. The Argonate protein in the RISC complex incorporates one of the more stable duplexes in the mature miRNA into the RISC complex to regulate the expression of the target mRNAs. The other chain is degraded or prepared to be thrown out of the cell. Some miRNAs are found packed in exosomes generated from multivesic substances. Other miRNAs can be exported in the presence of proteins that bind to "RNA". Again, some miRNAs can be extracellularly delivered via microvesicles during membrane blebbing. These miRNAs in the extracellular area can be taken up by other cells, disrupted by RNA, or removed from the body (Etheridge*et al*., 2011).

1.7. microRNAs and Cancer

microRNAs (miRNAs) are small non-coding RNAs. They have significant responsibilities in post transcriptional gene regulation. miRNAs organized by translational inhibition and unstablization of mRNA in animal cells (Bushati and Cohen, 2007).

MicroRNAs are contained in the organizing of almost all molecular mechanisms such as proliferation, development, cell fate, metabolism, apoptosis and cell death. In studies conducted, it was discovered that miRNAs and cancer are linked to abnormal expression levels. miRNAs have been reported to be therapeutic agents (Saito and Jones, 2006; Baytek, 2015).

Cancer is a multidisciplinary process. Cancer is when abnormal cells divide in an uncontrolled way with genetic changes. Cancer can expand along the all body and this is metastasis.Normal cells change their properties with genetic and epigenetic modifications. The new phenotypic cells that are exchanged gain different properties. Some of these features are cancer cells failure to undergo apoptosis, gain unlimited cleavage abilities, avoidance of inhibitory growth signals, and incresed of angiogenesis. The onset of progression and progression of the cancer cell regulates the genes that express cell proliferation, differentiation and apoptosis mechanism. Cancer cells act as tumor suppressor oncogenes by the effect of genes. Oncogene products can be classify into six groups. They can be transcription factors, chromatin remodelers, growth factor receptors, growth factors, signal transducers and apoptosis regulators (MacFarlane and Murphy, 2010).

Oncogenes play the role with change of genetic that boost the gene and modify promoters or enhancers to increase gene expression or modify of protein structure. On the contrary, tumor suppressor genes play roles in biological processes. If, lose of function of tumor suppressor genes occur in cell, the cancer process is occured. Currently studys, oncogenes and tumor suppressor genes is consist of protein coding genes to include miRNA (MacFarlane and Murphy, 2010).

miRNAs have a essential function in regulating most of cellular and metabolic pathways such as cells differantiation, survival, cells proliferation. miRNAs play the role as oncogenes or tumor suppressor genes. Dysregulation of miRNA expression profiles has been demonstrated in most tumors examined. But the especially classification of miRNAs can be

diffucult which separate function as oncogenes or tumor suppressor genes.. Because, expression of mirna is complicated subject. miRNAs can not be cause of cancer direct since they play a role indirect action in cellular phenotyope. Furthermore only one miRNA can be regulate multiple genes. For all, miRNAs are secret agents of cancer development and they success with oncogenes or tumor suppressor role (MacFarlane and Murphy, 2010).

1.7.1.Mechanisms of microRNAs Dysregulation In Cancer

Currently studies have shown that expression of miRNAs is dysregulated in human malignancies. This changes mechanisms can be caused by chromosomal abnormalities or by epigenetic silencing or by dysregulation of transcription factors targeting specific microRNAs and biological defects in the miRNA. This modifications in genomic miRNAs are happened with amplification or deletion in miRNA genes (Croce, 2009; Peng and Croce, 2016).

The changes in miRNA expression were found to be significant when cancer and normal cells were compared. Overexpression or knockdown of miRNA genes give rise to cancer process.miRNA in tumorigenesis contribute to development the oncogenesis and loss of differentiation with dysregulation of multiple protein coding oncogenes or tumor suppressor genes(Croce, 2009; Manikandan*et al*., 2008).

1.7.2.microRNAs as Tumor Suppressor Genes

Some miRNAs are downregulated in cancer cells in oncogenesis. These types of miRNAs are considered tumor suppressor genes. Tumor suppressor miRNAs generally prevent tumor development by negatively inhibiting oncogenes and/or genes that control cell differentiation or apoptosis. These miRNAs is encoded as TS-mir (Blandino*et al.*, 2014). At present, various miRNAs are considered as tumor suppressor genes, for example, miR-15a and miR-16–1 (Manikandan*et al*., 2008).

1.7.3.microRNAs as Oncogenes

miRNAs are synthesised as overexpressed in tumor. Most of them function oncogenic except a few. These oncogene miRNAs, encoded as “oncomirs”. Oncomirs inhibit tumor suppressor genes. They act by affecting cell differentiation, proliferation and apoptosis genes negatively. They try to inhibit any pathway that prevents the cancerous process. The studies underline the fact that miRNAs function as oncogenes or tumor suppressor genes. For example, it has been determined that overexpression of mir-17-92 increases the formation of B-cell lymphomas at a substantial rate (Manikandan*et al*., 2008).

1.7.4.The microRNAs in Body Liquid

The importance of microRNA (miRNA) in cancer cells has been recognized in recently. Appropriate control of miRNA expression is necessary for maintaining a fixed state of cellular machinery. The miRNAs of the blood of both healthy and diseased individuals were detected. These miRNAs are found in lipid-lipoprotein structures. For this reason, the miRNAs have a stable structure. Their presence in body fluids also allows them to act as diagnostic markers. Despite this discovery, it is unclear how the functions of the miRNAs are made. For this reason, more research and study are required (Kosaha*et al.*, 2010; Kwak*et al.*, 2010).

In one study, the presence of serum miRNAs was found by Solexa sequencing. In this study, healthy controls were performed in lung cancer patients, colorectal cancer patients and diabetic patients. No disease specific miRNAs were detected in sera of healthy control groups. This finding has been associated with the possibility that miRNAs specific to these diseases may be present in relevant patient sera in humans. As a result, it has been shown that extracellular miRNAs can be used for diagnosis of cancers by analyzing them (Chen*etal*.,2008).

Unlike RNAs between different liquid types, miRNAs exhibit stable and distinctly expressed expression profiles (Weber*et al*., 2010; Zubakov*et al*., 2010). The miRNAs in the context are resistant to RNAi ", pH" and high temperature variability in serum-free serum-like body fluids (Chen*etal*.,2008). Human miRNAs circulating in body fluids are primarily important for the detection of cancer and presence in serum and plasma. In the human body

fluids such as plasma, urine or breast milk, 200-500 miRNAs have been detected with qPZR tecnique. Detection of miRNAs in body fluids is a promising noninvasive diagnostic method for the diagnosis of cancer patients (Weber*et al*., 2010).

Although the mechanistic mechanism of miRNAs is not yet explained, it is predicted that the tissues will passively passively seep into the circulation. It has been determined that miRNAs in body fluids originate from lipid vesicles, microvesicles or exosomes. Microvesicles are released from the cell by blistering. The sizes are between about 100 nm and 1 micron. The exosomes are small vesicles about 30-100 nm in size. It occurs as a result of fusion of multivesicular structures composed of endosomes with plasma membrane. They are found in body fluids like plasma, serum, saliva, breast milk and urine. Exosomes can be secreted from various cells such as epithelial cells, T and B cells, dentric cells and cancer cells (Ramachandran and Palanisamy, 2012).

High levels of mir-21 in serum of B cell lymphoma patients, the first studies on the modulation of miRNAs, have been associated with survival rates of individuals who have not recurred the disease (Lawrie*et al.,* 2008). It has also been strongly associated with patients with elevated levels of mir-21 in serum or plasma, lymph node metastasis, advanced clinical stage, and poor survival prospects in NSCLC (Liu*et al*., 2012).

In another study, serum levels of mir 10b and -34a in the sera of breast cancer patients were decreased, while serum levels of mir-195 and let7a were increased (Roth*et al.,* 2010; Heneghan*et al*., 2010). It has been shown that urinary samples from body fluids can be used to determine the presence of bladder cancer levels of mir-126 and mir-182. In saliva, decreased levels of mir-125a and mir-200a were associated with squamous cell carcinomas of the oral cavity (Park*et al*., 2009).

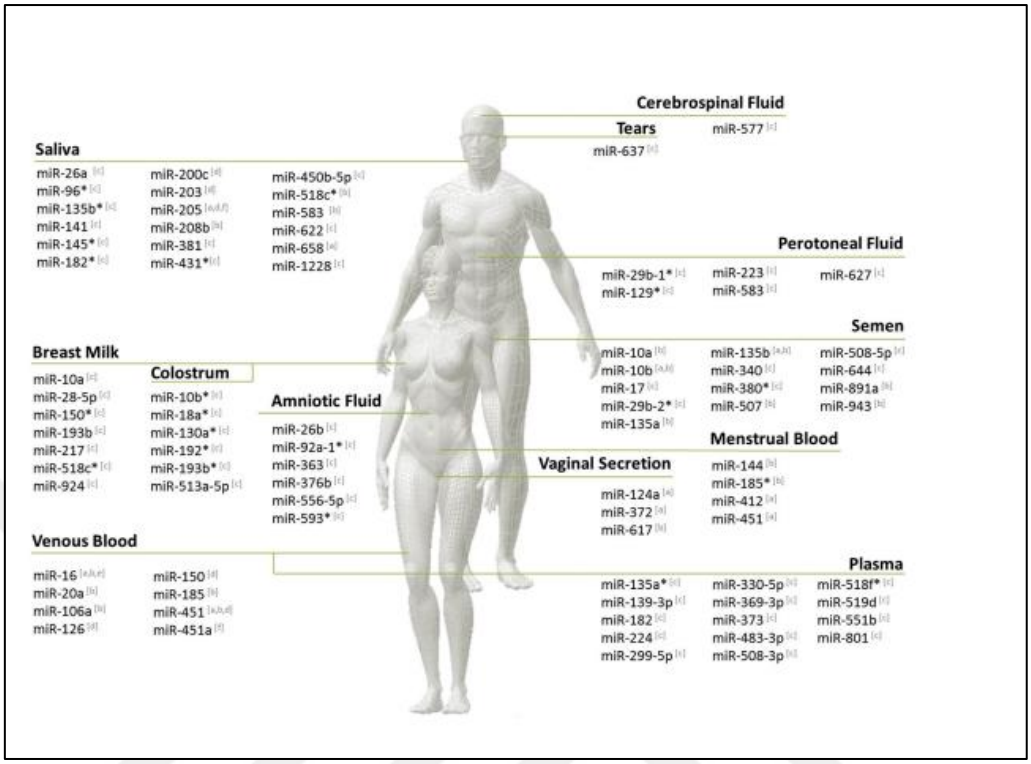


Figure 1.6: microRNAs in body liquids. It is adapted from (Silva*et al*., 2014).

1.8.microRNAs Associated with Squamous Cell Laryngeal Cancer

Nowadays, detecting or regulating increased or decreased miRNA expression is of great importance in cancer pathology. For this reason, different methods such as antisense inhibitors, transgenics, specific promoters, real-time PCR and miRNA microarray have begun to be used when studying the function of miRNA (Zhang*et al.*, 2006; Jiang*et al*., 2005).

Certain miRNAs have an effect on genetic changes in tumors.A number of expression profiling studies have been conducted in which miRNAs show a significant decrease or increase in expression levels at significant ratios (Chen*et al*., 2014). (Tablo X)

Increased Expression:

miR-423, miR-93, miR-106b, miR-16, miR-20a, miR-155, miR-193a, miR-25, miR-18b

miR-92, let-7i, miR-17-5p, miR-19b, miR-223, miR-27a, miR-142-3p, miR-210

miR-106a, miR-15a, miR-21, miR-29b, miR-130b, miR-205, miR-422b, miR-181a

miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221,miR-193b

Decreased Expression

miR-125b, miR-375, let-7a, miR-10a, miR-140, miR-100, miR-143, miR-99a,

miR-30c, miR-365, miR-127, let-7c, let-7e, miR-199b, miR-26a

Figure 1.7 : Expression-altering miRNAs in Plasma of Patients with Squamous Cell Laryngeal Cancer.It is modified from (Ayaz*et al*., 2013).

2.MATERIALS and METHODS

2.1.Equipment and Devices

2.1.1.Equipments

- Nanodrop spektrofometre (Thermo Scientific-ND 2000c, Germany)
- Laminar air-flow (Class II Safety Cabinet) (Metisafe, Turkey)
- Micropipette (Thermo Scientific, Germany)
- Refrigerator (4 °C) (Vestel, Turkey)
- Deepfreeze (-20°C) (Vestel, Turkey)
- Deepfreeze (-80°C) (Wisd, DAIHAN Scientific, Korean)
- Centrifugal (Hettich Retina 420R, Germany)
- Mini Centrifuge (Thermo Scientific, Germany)
- Thermal Cycler (PCR) (Techne-TC 5000, ABD)
- Vortex(Wise Mix-VM10, Korean)
- Spin Down
- 0.2-1.5 ml Eppendorf (Nest Biotechnology, China)
- LightCycler ® Plates - Roche Applied Science (Basel, Swiss)
- Roche LightCycler 480 (Basel, Swiss)
- xCelligence Device and Equipment
- Other consumables needed for Molecular Genetic applications

2.1.2. Solutions

- TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, ABD)
- TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, ABD)
- TaqMan miRNA Assay (Applied Biosystems, Foster City, CA, ABD)
- Trizol, İsopropanol, Chloroform, 75% Ethyl Alcohol Solutions7
- X-tremeGENE™ siRNA Transfection Reagent
- OptiMEM

- MISSION® microRNA Mimic (hsa-miR-203, miR-26b, miR-200c-3p, miR-203 miR-363-3p and miR-1825)
- Matrigel Matrix (Corning, NY, USA)

2.1.3.Computer Programs

- xCelligence Software RTCA DP

2.2.Methods

2.2.1.Cell Culture

Hep-2 cell lines were cultured in RPMI-1640 (Gibco-BRL, Bethesda, MA) medium, supplemented with 10% fatal bowine serum (FBS) and 1% Penicillin Streptomycin Amphotericin (PSA) in a 5% CO^2 humidified incubator at 37 °C. The cell lines were passaged after reaching 70% confluency.

2.2.2.Transfection Of Hep2 Cells

Hep-2 larynx cancer cell line was used in experiments. miR-26b, miR-200c-3p, miR-203 miR-363-3p and miR-1825 mimics were provided for use in *in vitro* studies performed and were transfected into the Hep-2 cells. Expression of these miRNAs in the cells was demonstrated by real-time PCR amplification following transfection. Mature miRNAs were obtained from SigmaAldrich using a control miRNA from SwitchGear Genomics, which is an oligonucleotide sequence that does not target any gene as a control.

Cells were grown in 6 well plates to 25000 cells per well. When cells were arrived 60-70% confluency, miR-203, miR-363-3p, miR-26b, miR-200c-3p and control miRNA were transfected using X-treme GENE siRNA Transfection Reagent.

For per well in plate 2.5µl transfeciton reactive with 250 µl Opti-MEM nutrient, in other tube 10pg mimic miRNA 250 µl Opti-MEM were mixed and waited room temperature 5 minutes. Subsequently, these two mixture by combinedand incubated 20 minutes, and thencells were

treated with the appropriate volume of transfection mixture in the plates used and left at 37 ° C for 1 hour. Later, 500 µL of Opti-MEM was added to each of the plates to allow the cells to incubate at 37 ° C. Cells were used for functional studies 24,48 and 72 hours .

2.2.3.RNA Isolation

Grown cells on plates were plates removedfrom mediums. Plates were washeds with 1ml PBS and cells scraped with cell scraper from plates. Washeds cells with 1ml PBS were transferreds into ependorf tubes. Samples were homogenized by pipettinge in Trisol solutions. This mixture waiteds for 10 minutes at room tempreture for the tissues to be disintegrated thoroughly then proteins wll be bound to the nucleic acids and completely separated from the nucleic acids. Then, 200µL chloroform addeds and the mixture kept as 2-3 minutes at room temperatures. The mixture was centrifuged at 12000g for 15 minutes at 4 ° C. After phase separation, the upper RNA-containing phases we transferred to fresh microcentrifuge tubes. 500 ml of isopropanol was added to new tubes and they incubated for 10 minutes at room temperature then the tubes were centrifuged at 12000g for 10 minutes at 4 ° C. After upper phase separation, RNAs was washed with 75% ethyl alcohol and is centrifuged at 7500g for 5 minutes at 4° C. The supernatant part wasremovedandthe RNA pellets were expected to dry out. Dryed RNAs are dissolved by adding nuclease-free water.

2.2.4.cDNA Synthesis

qRT-PCR assays performed using TaqMan probes.Total RNAs from the samples were diluted to a concentration of 15 ng / µl and 2 µl of each sample was used. miRNA cDNA synthesis was performed according to the manufacturer's instructions using the miRNA RT primers in the loop construct and the "Taqman Reverse Transcriptase Kit". (Table 2.1)

Component	**Amount (µL) for each RxN**
DNTP	0.075µL
10x RT Buffer	0.75µL
RNAse Inhibitory	0.94Ml
dH2O	2.581µL
Reverse Transkriptase	0.5µL
miRNA RT Primer	1.5µL
RNA	2µL

Table2.1: Tagman Reverse Transcriptase Kit Prosudure

Temperature	**Time**
16°C	30 Minutes
42°C	30 Minutes
85°C	5 Minutes
4°C	∞

Table 2.2 : PCR Condition for cDNA Synthesis

2.2.5.qRT-PCR

qRT-PCR assays were performed by using TaqMan assay. TaqMan miR-26b, miR-200c-3p, miR-203, miR-363-3p, miR-1825 and RNU43 probes used as controls were purchased from Applied Biosystems (Foster City, CA). qRT-PCR procedures were performed using the LightCycler480-II real-time PCR device with the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) kit followed by the manufacturer's protocol. RNU43 from small

nuclear RNAs was used as a control and experiments were duplicated. Results were evaluated by delta delta ct method with student's t-test.

Component	**Amount (µL)**
Universal Master Mix	5µl
dH2O	2,5µl
Probe RT	0.5 µl
cDNA	2 µl

Table 2.3 : Tagman qRT PCR Protocol

The prepared mixtures were subjected to real time PCR operation using the Roche LightCycler 480 instrument according to the conditions given in Tables X below.

Programme	**Temperature**	**Time**	**Cycle**
Preincubation	95°C	10 minutes	1
Amplification	95°C	15 second	40
	60°C	40 second	
	72°C	1 second	
Cooling	95°C	30 second	1

Table 2.4 : qRT-PCR Conditions

2.2.6.Identification of miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p Proliferation in Hep-2 Cells

The activity of miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p was determined by performing cell proliferation test in the Hep-2 cell line. miRNAs enabled us to

evaluate the effects more effectively and efficiently on cell proliferation using the xCELLIGENCE System device.

With xCELLIGENCE system, cellular events can be analyzed in real time. This system is installed in an incubator and measures the electrical impedance by means of the microelectrodes located on the ground of the E-plates where the cells are planted. Impedance measurement provides quantitative information about the biological state of the cells, including cell number, viability, morphology and movement.

Cells were plated into 16-well e-platelets in three replicates with 15,000 cells per well. Twenty-four hours later, cells were subjected to proliferation changes by taking a measurement every 15 minutes after transfection with miRNA mimics and for three days and in a xCELLIGENCE instrument providing simultaneous analysis.

2.2.7. Identification of miR-203 and mir-363-3p Migration and Invasion in Hep-2 Cells

Invasion and migration changes of mir-203 and mir-363-3p treated cells, which were found to be significantly altered in accordance with the proliferation results, were performed in the CIM plate wells of the xCELLIGENCE system.

The migration test was performed by adding 15,000 'cell mix with Fetal Bovine Serum (FBS) free medium. Median migration potentials of FBS, which were from the chemoattractant membrane through the membranes and below the plate surface, were measured with the aid of a device for 24 hours.

For the invasion test, Matrigel Matrix (Corning, NY, USA) - Tris HCl coating buffer mixture, prepared in a 1: 4 ratio in CIM plate wells, was added to 120 µl wells.Fetal Bovine Serum (FBS)-free medium and 15,000 cells per well on Matrigel incubated for 4 h at 37 ° C. Cells were passed through Matrigel to measure the median invasion potentials of the FBS from the chemoattractant and below the plate surface for 24 hours (TableX).

Component	**Amount**
Matrigel	1X
Tris HCL	4X

Table 2.5 : Matrigel Matrix Preparation

Thus, the effects of miRNAs on migration and invasion potentials on cancer cells were determined.

2.2.8.Soft Agar Colony Assay

miR-203 and miR-363-3p transfected with Hep-2 cells and it was collected 24 hours later then prepared for soft agar. For the soft agar mixture, 1.2% low melting agar (0.36 g) and 30 ml H_2Omixture were first heated in the microwave for 2 minutes. The mixture was kept 40 ° C water bath for freezing to 50 ml of falkonite. The mixture containing 2X RPMI medium was prepared with 20% FBS and 2% PSA in a total volume of 40 ml was maintained at 40 ° C water bath for 30 minutes. The two mixtures were dispensed in equal volumes (15 ml + 15 ml) to 6 wells plates as 1 ml per well. It was then allowed to cool down for 1 hour at room temperature.

Cells transfected with miRNA and control miRNA were trypsinized and counted. Cells were counted as 3,000 cells in each well were prepared to be plated on 6-well plates as a 3-repetition.

Medium (0.6 g agar + 30 ml H2O) containing 0.6% agar + 10% FBS was heated in the microwave for 2 minutes to prepare the upper agarose mixture and then allowed to stand for half an hour at 40 ° C in water bath.The cells and this mixture were added to the pre-warmed agar as 1 ml in total and the mixture was allowed to cool at room temperature for about 1 hour. Medium was added with 2 ml of 10% FBS, 2% PSA, media were changed every 2-3days, followed by 2-3 week follow-up of the cells. The fixation and staining were carried out in a mixture of 0.01% crystal violet, 10% ethanol were dissolved in distilled water. Colonies were counted after washing 3 times with distilled water to remove background dying.

2.2.9. Statictical Analysis

Statistical analyzes were performed on the log-transformed data using "two-sided Student's t test" . P-value <0.05 was accepted statistically significant. Error bars were drawn using ± standard error values. Standard error values were obtained by dividing the sample number of standard deviation values by the square of the number of samples then evaluations of working microRNAs were obtained (172).

3.RESULTS

3.1.RNA Isolation

The transfected microRNAs were isolated with trisol, and then their concentrations were measured using a nano-drope device. The measured values are the same as on the table.

miRNA	**RNA Concentrations**
miR-26b	325 ng/µl
miR-1825	425 ng/µl
miR-203p	441 ng/µl
miR-363-3p	335 ng/µl
miR-200c	250 ng/µl
Non-target miRNA	505 ng/µl

Table 3.1: Concentrations of miRNAs

3.2.qRT-PCR

Result of qRT-PCR were evaluated by delta delta ct method with student's t-test. The qRT PCR image of mir-203, mir-363-3p,mir200c,mir-1825 and mir26b transfected into Hep-2 cells. This table shows that the transfection has been successful.When evaluated according to non-target, the peak at high rates indicates success.

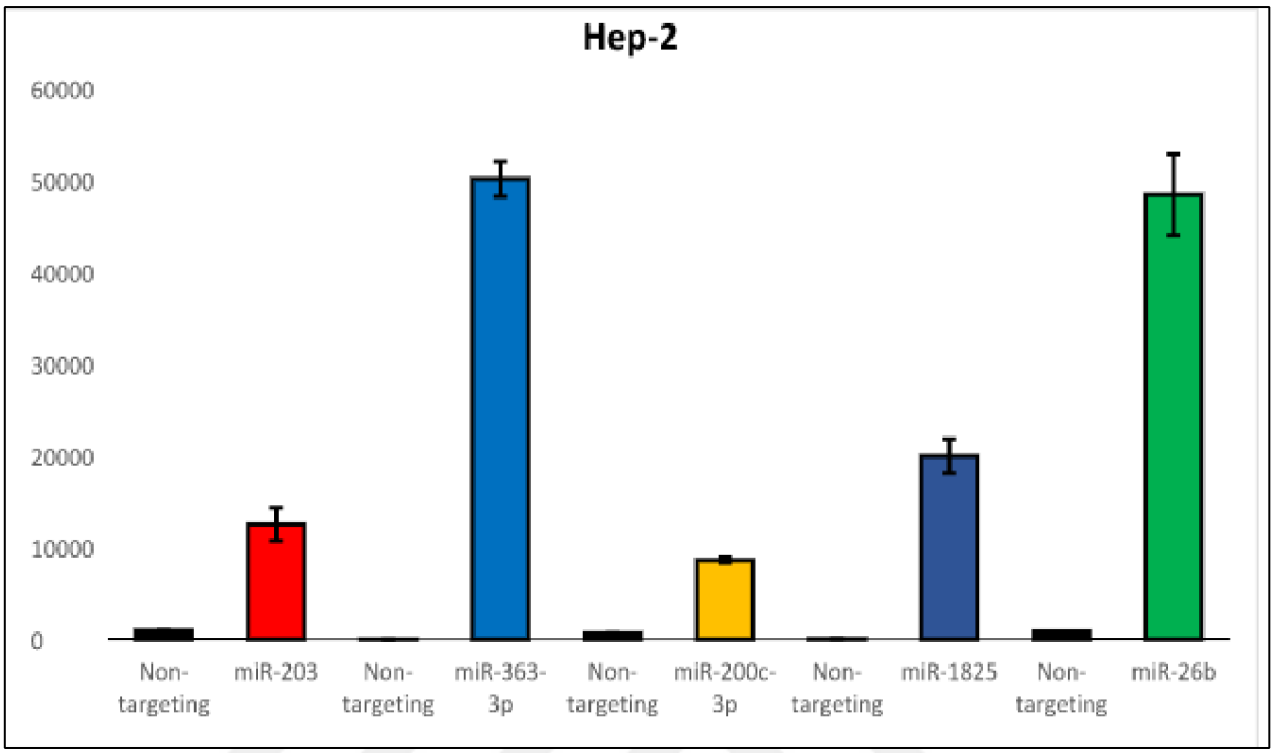


Figure 3.1: The qRT PCR image of mir-203, mir-363-3p,mir200c,mir-1825 and mir26b transfected into Hep-2 cells.

MiRNAs	**t-test**
miR-26b	0,03
miR-1825	0,0068
miR-203p	0,09
miR-363-3p	0,007
miR-200c	0.0002

Table 3.2 : After qRT-PCR, p values of delta t-test of miRNAs

3.3.Identification of miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p Proliferation in Hep-2 Cells

In xCELLIGENCE System device at the end of the measurement, proliferation levels of the mir-203 and miR-363-3p transfected cells was determined according to the control miRNA. (miR-203 p: 0.0007 and miR363-3p p: 0.001) The result of these miRNAs acting as tumor suppressor are the ones we expected. No significant change in the proliferation capaticities of miR-26b,miR-200c-3p and miR-1825 was observed. (miR-1825 p: 0.1). Those remaining below the non-target graph indicate that the proliferation is slower. The remaining ones indicate an increase in proliferation. Accordingly, it can be interpreted that miR-1825 has an increased proliferation. It can be said that mir-203 and mir363-3p also decreased proliferation. According to the results, migration and invasion assays of miR-203 and MiR363-3p, which resulted in a significant proliferation assay, were continued.

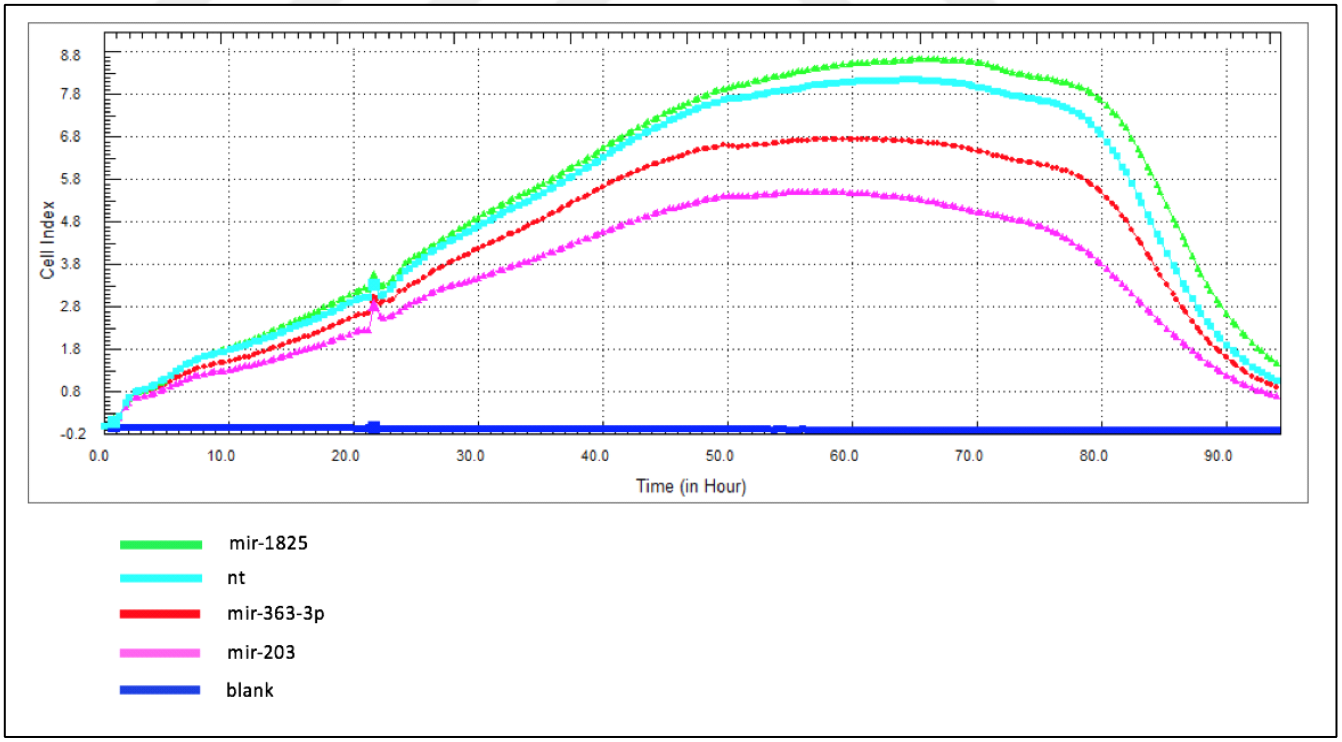


Figure 3.2 : Proliferation graphic belonging to mir-1825 , mir-363-3p and mir-203 on Hep-2 cells

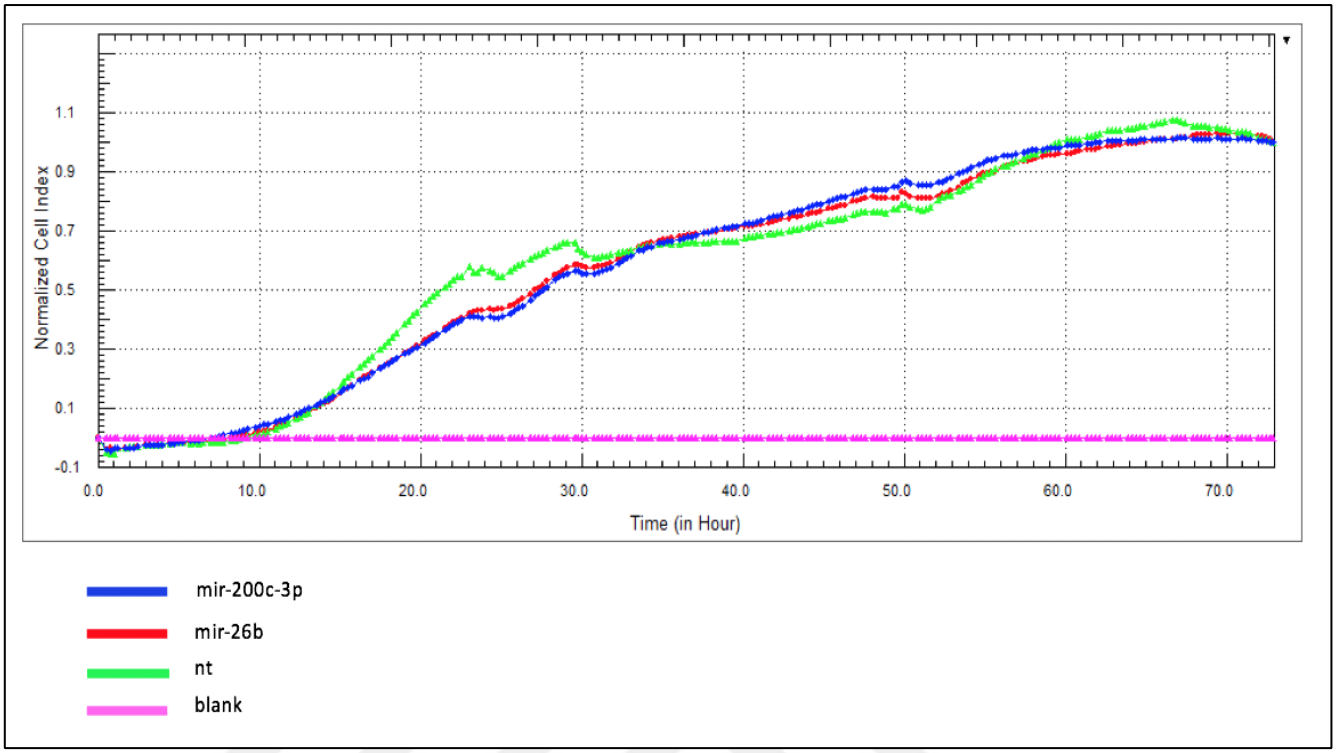


Figure 3.3: Proliferation graphic belonging to mir-26b, and mir200c -3p on Hep2 Cells

MiRNAs	**t-test**
miR-26b	0.2
miR-1825	0.1
miR-203p	0.0007
miR-363-3p	0.01
miR-200c	0.09

Table 3.3 : After proliferation, p values of delta t-test of miRNAs

3.4.Identification of miR-203 and mir-363-3p Migration and Invasion in Hep-2 Cells

Invasion andmigration changes of mir-203 and mir-363-3p treated cells, which were found to be significantly altered in accordance with the proliferation results, were performed in the CIM plate wells of the xCELLIGENCE system. Migration of mir-203 and mir-363-3p in Hep-2 cells were found to decreased significantly in relation to normality. According to findings, these miRNAs acted like tumor supresor.

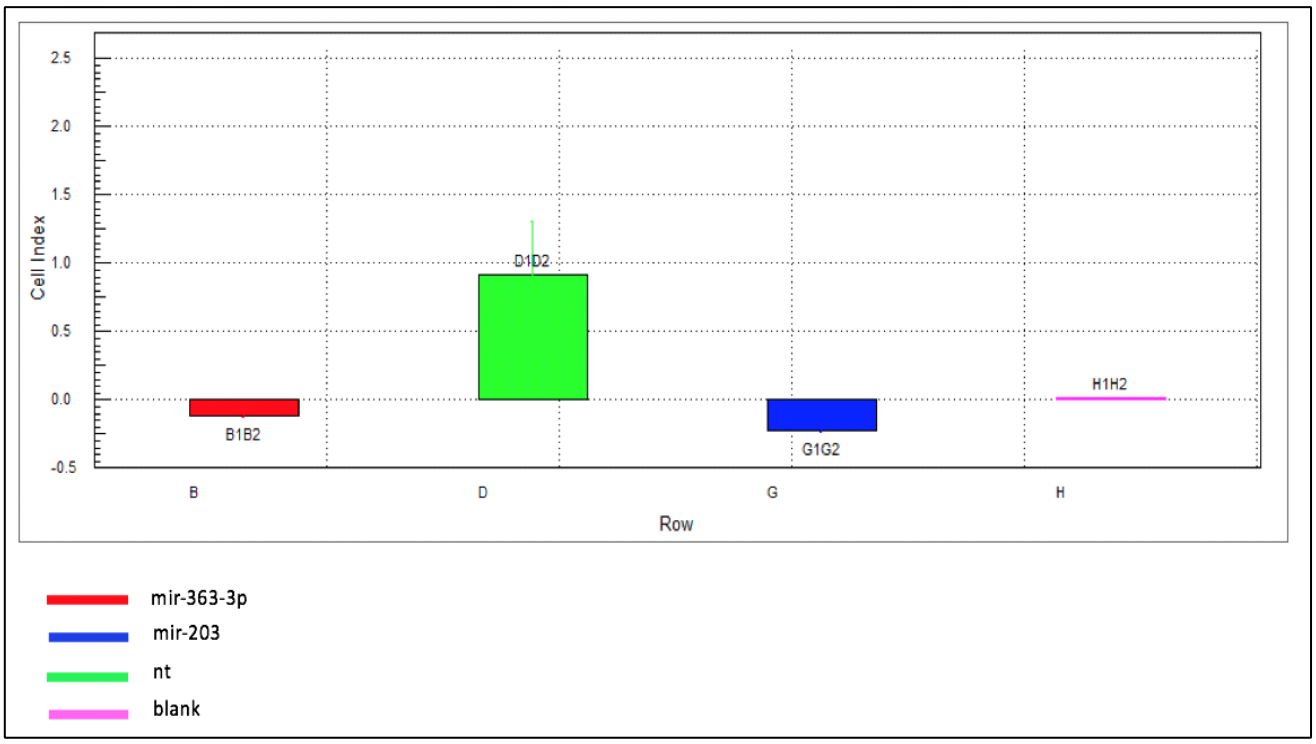


Figure 3.4: Migration graph of mir-203 and mir-363-3p in Hep-2 cells

MiRNAs	**t-test**
miR-203	0,0007
miR-363-3p	0,01

Table 3.4 : After migration assay, p values of delta t-test of miRNAs

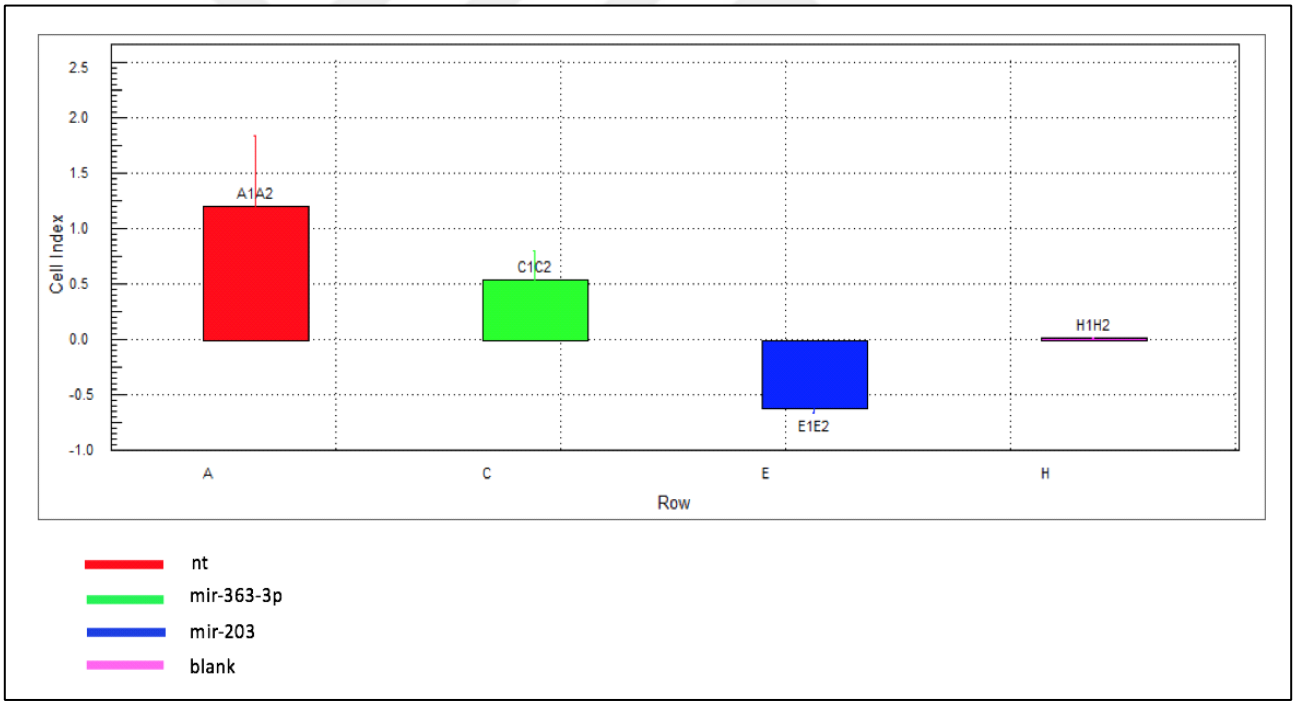


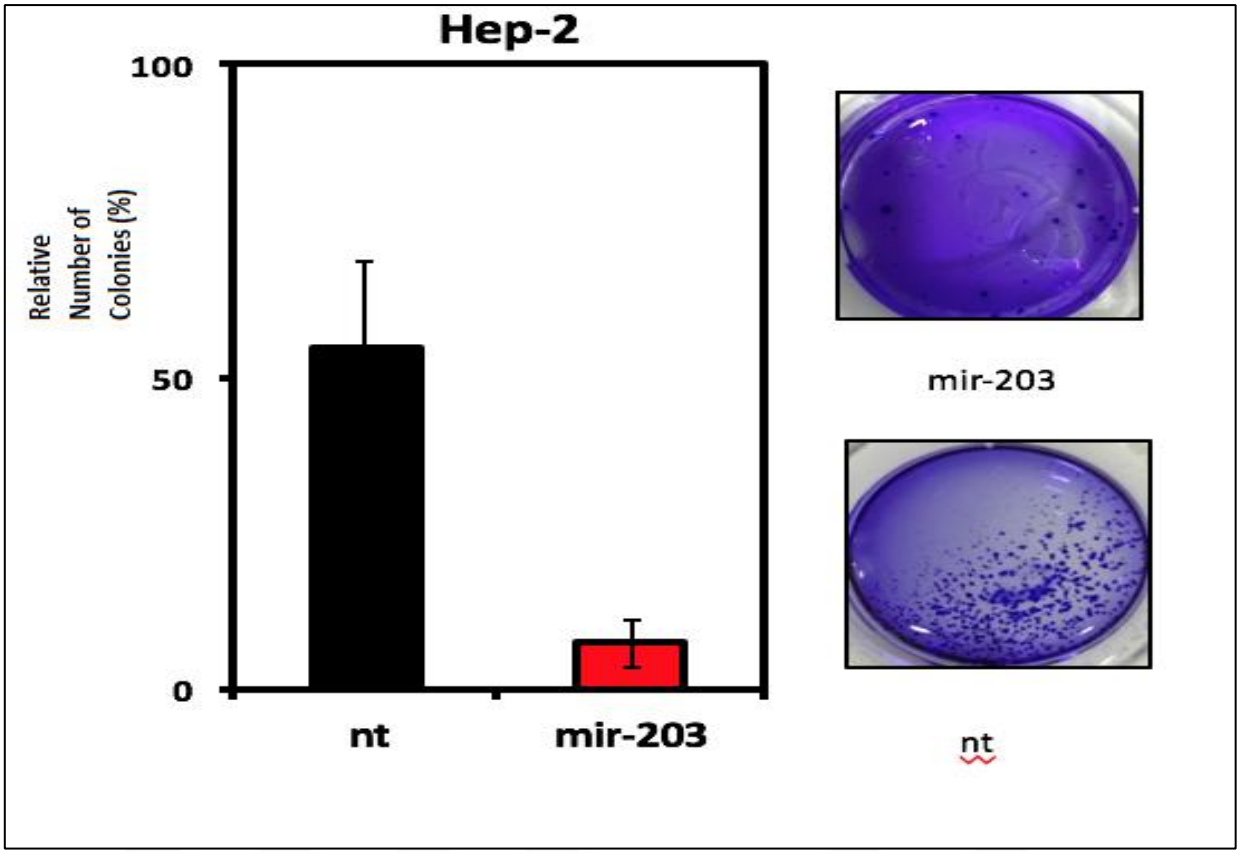
Figure 3.5 : Invasion graph of mir-203 and mir-363-3p in Hep-2 cells

MiRNAs	**t-test**
miR-203	0,008
miR-363-3p	0,01

Table 3.5 : After invasion assay, p values of delta t-test of miRNAs

3.5.Soft Agar Colony Assay Results

In the study, Hep-2 cells were transfected with mir-203 and mir-363-3p to form colonies. It was determined that the ability of Mir-203 and miR-363-3p transfected cells to compare with the control group resulted in a reduction in the ability to form colonies. This significant reduction shows that mir-363-3p and mir-203 are from miRNAs that are tumor suppressor. In these step-by-step analyzes, we observed the effect of miRNAs on Hep-2 larynx cancer cells compared to normal. As a result, we found that mir-363-3p and mir-203 were significant.


Hep-2
100
50
0
Relative Number of Colonies (%)
nt
mir-203
mir-203
nt

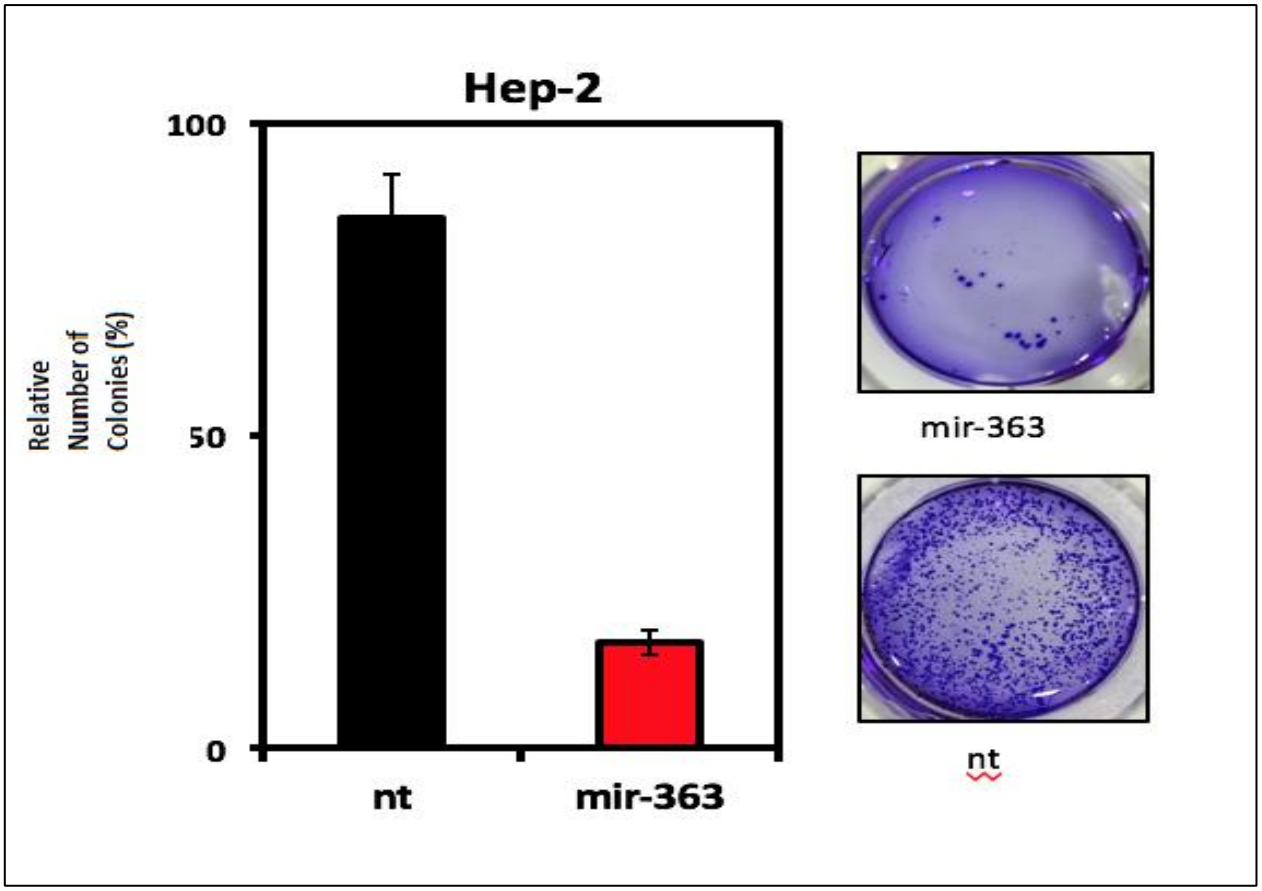


Figure 3.6 : Colony Assay images and graph of mir-203 and mir-363-3p in Hep-2 cells

MiRNAs	**t-test**
miR-203	0,002
miR-363-3p	0,009

Table 3.6 : After soft agar colony assay, p values of delta t-test of miRNAs

4.DISCUSSION

Initial observations about the mechanisms of cancer formation are that tumor mass from a homogenous clone of a single mutated cell is formed. Today, however, cancer stem cell theory suggests heterogeneous cell populations within the tumor that are different in terms of tumorigenic potential. Cancer stem cells, which are similar to stem cells that provide tumor initiation and continuation, may be resistant to chemotherapy and radiotherapy, explaining why today's treatments often do not completely destroy the tumor (Reya*et al*., 2001).

Laryngeal cancers are among the most common types of cancer in men, accounting for 45% of the major cancers. The majority of malignancies of the larynx are squamous cell carcinomas of epithelial origin (90-95%) (Farhadieh*et al*., 2009). The incidence of LSHD has shown a decline trend in the last 35 years worldwide. However, the decline in mortality rates is not that grate. The incidence of laryngeal cancer and mortality rates due to this cancer are predicted to increase in our country in the coming years (SEER Cancer Statistics Factsheets: Larynx Cancer, 2011). The incidence of male to female ratio of larynx cancer is about 10: 1. However, in recent years women have increased incidence in women with increased smoking. The ratio of male to female in Europe and the US has changed by about 5: 1 (Braakhuis*et al.*, 2014; Pac, 2013).

Therapeutic methods for the treatment of laryngeal cancer are very limited due to the complex of laryngeal cancer. Treatment for laryngeal carcinoma has been developed in early and non-metastatic cases. However, studies for treatment in advanced cases continue to be made (Wang*et al.*, 2013; Xu*et al*., 2013).

In addition, one of the most important problems in laryngeal cancer is the problem of recurrence of cancer in patients after laryngectomy procedures. Cells responsible for cancer recurrence are a small group of cells within total cancer tissue. For this reason, it is very difficult to investigate genetic changes in recurrent cells to solve this problem and the number of studies that can be done is very less (Wang*et al.*, 2013; Xu*et al*., 2013).

The mechanisms of tumor-initiating stem cell-like cells must be well understood. If this is achieved, it will be possible to develop better and more effective supportive treatments that will increase the patient's life span. Understanding the underlying mechanisms of the

pathogenesis of laryngeal cancer will be a crucial step in the development of new and effective therapeutic approaches (Wang*et al.*, 2013; Xu*et al*., 2013).

There is now a great deal of evidence showing that miRNAs play a role in laryngeal carcinogenesis (Wang*et al.*, 2013; Xu*et al*., 2013). MicroRNAs (miRNAs); protein-coding and single-stranded RNA molecules (Zhang *et al.*,2006;Lund*et al*., 2013). The number of miRNAs that are about 20-22 nucleotides in length exceeded 1000 in humans (Gregory*et al*., 2005). MiRNAs have been associated with cell growth, proliferation and cell apoptosis, and regulatory effects have been reported in almost every cellular process, depending on location and time-varying expression. Recent studies have shown that miRNA irregularity plays an important role in the formation of human tumors in many physiological processes including cancer pathogenesis, cancer initiation, progression, metastasis and invasion (Iorio and Croce, 2009; Baranwal and Alahari, 2010).

MiRNAs have been first discovered in 1993 by identifying that lin-4, a short, non-coding RNA gene in Caenorhabditis elegans, controls nematode development by suppressing the accumulation of lin-14 protein (Lee et al., 1993; Olsen and Ambros, 1999). In the following years, it has been suggested that gene expression regulation through miRNAs may be a common regulatory model by the detection of let-7 in many living species (Lagos-Quintana*etal*., 2001;Nikitina*et al.*, 2012).

In many studies, MiRNAs have been reported to act as tumor oncogenes or tumor suppressor genes (Filipowicz*et al.*, 2008). After understanding the important effects of miRNAs in the carcinogenesis process, changes in expression levels of miRNAs in specific cell types in different cancer types have been investigated. As a result, miRNAs differed between normal and pathological tissues. miRNA encoding genes were found to be intense in the fragile regions. When the excess of genomic regions associated with the cancer or more than half of the fragile regions emerged from genes encoding miRNAs, miRNAs were noticed their role in the cancer pathogenesis (Calin*et al*., 2004). By detecting that miRNAs regulate gene expression, miRNA levels were compared between cancer cells and normal cells. This process has gained great importance in terms of diagnosis, follow-up and treatment. The identification of the functions of the miRNAs and their roles in cancer promises to elucidate

the molecular pathology of cancer and to develop treatments for molecular targets (Johnson*etal*., 2005).

Microarray profiling methods are important for determining miRNAs with different expression levels in different tissues and cells. Detection of miRNAs expressing significant expression by microarray studies allows for the elucidation of disease pathogenesis using various in silico, in vitro and in vivo tools. The possible targets of miRNAs identified should be determined primarily by the use of bioinformatics tools. ectopic expression of miRNAs in vitro and in vivo should be investigated following expression changes in these targets. And it must go through both the transcriptional level and the translational level validation process. These are important functional methods for elucidating the role of miRNAs in carcinogenesis (Zhao*et al.,* 2013; Xu *et al.,* 2013).

Mature miRNAs are not those with complex structure. They do not undergo a known modification. These properties provide an advantage in that expression levels can be easily detected. At the same time miRNAs produce tissue-specific expression profiles. It shows the high degree of conservation of miRNA sequences between human and model organisms. Stabilites are much higher than mRNAs due to the shortness of miRNAs (117,118). All these properties make strong candidates for miRNAs to be used as biomarkers and for characterization of physiopathological conditions (Weber*et al.,* 2010; Park*et al.,* 2009).

The first study to show the relationship between miRNA genes and cancer was performed by Calin *et al.* (Calin *et al*., 2002). In the study, it was stated that 13q14 deletions seen in more than half of the CLL patients may be responsible for miR-15 and miR-16 genes. In addition, differences in miRNA expression levels of solid tumors and normal tissues have been shown (Michael*et al*.,2003) and in the same way breast cancer (106), Burkitt's lymphoma (Metzler*etal*., 2004; Zhang*et al*., 2006), malignant brain tumors (Chan*etal*., 2005), prostate (Sevli*etal*., 2010) and changes in miRNA levels in bladder and colon cancer (Lamy*et al.,* 2006).

In addition to the tissues, miRNAs have also been found to circulate (Mitchell*et al.,* 2008). It is believed that miRNAs found in body fluids are protected from RNAs (Valadi*etal*., 2007; Michael*et al*., 2010), trapped in exosomes or interacting with Argonaut-2 proteins

(Turchinovich*etal*., 2011), and that amounts of body fluid miRNAs do not change up to 48 hours at room temperature, solvation procedures did not alter miRNAs' stability (Blondal*etal.*, 2013).

The miRNAs found in serum, saliva and urea have been associated with many pathologies including cancer. In addition, colostrum, tear, amniotic fluid, semen, cerebrospinal fluid andprostate fluid were also found (Sun*et al*., 2013; Ghali*et al.*,1990; Montenegro*et al.*, 2009; Machida*et al.*, 2013; Guzel*et al*.,2014). Circulating miRNAs exhibit a strong discriminatory character in various diseases (Weber*et al*.,2010).

The presence of miRNAs in circulation was first suggested by the presence of miR-209 and miR-499 in the serum of patients with acute myocardial infarction (Corsten*et al.*,2010), suggesting the use of miR-1 as a new biomarker in diagnosing acute myocardial infarction (Ai*et al*., 2010).

In a study with urine specimens of patients with bladder cancer, expression levels of miR-200a, miR-200b, miR-200c, miR-141, miR429, miR-192 and miR-155 were lower than control groups. It was observed that the miR-200c and miR-141 levels remained at the same level as the control group and that the miR-200b, miR-200b and miR-429 levels remained stable after the operation (Wang*et al.*,2012).

Studies in the sera of patients with oral cancer suggest that the levels of miR-163 and miR-29a are decreased in the levels of miR-338-3p, miR-223, and miR-29a compared to sera from healthy controls (Blondal*et al.*, 2013; Sun*et al.*,2013).

In an miRNA expression comparison study; In 106 bladder tumors and 11 normal urothelia, the expression of 290 miRNA was compared and miR133b was found to be more prominent in normal urothelium compared to low grade urothelial carcinoma. It has been reported that miRNA changes can be used to predict tumor progression. Taking these changes into consideration, it has been reported that RNA-based therapy can inhibit the growth of cancer cells (Akao*et al.*, 2010).

Rather than examining any gene in cancer alone, the general analysis of gene expression has gained importance from a diagnostic point of view, and the use of microarrays has allowed tens of thousands of genes to be examined at the same time. By comparing gene expression profiles of different tumors, a molecular classification method has been developed. Such

studies show that gene expression profiling is informative about the dissimilarity of similar tumors and the clinical course of the disease as well as the response to treatment administered (Zhang*et al.,* 2006).

In a study of squamous cell head and neck cancer (HNSCC) patients, it was confirmed that miRNAs have different expression levels according to the healthy control group. In squamous cell head and neck cancers; it has been shown that the expression levels of miR-1, miR133a, miR-133b and miR-206 are decreased most frequently. In addition, expression levels of miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR- increase. Some miRNAs are found in more than one region between head and neck regions (Nohata*et al*., 2013).

Another study found that MiR-21-5p / let-7a and miR-34c-5p play an important role in LSCC carcinogenesis. The ratio of miR-21-5p / let-7a has been suggested to have an important clinical diagnostic potential to distinguish tumor tissue from normal tissue. miR-let-7a levels have been predicted for lymph node metastases and miR-34c-5p has been concluded to be a critical biomarker for patient outcomes (Kwak*et al.,* 2010).

The aim of our study was to identify miRNAs that could be associated with squamous cell laryngeal cancer. This will help us in the development of diagnostic and therapeutic methods of the disease with further analysis. For this purpose, we have carried out further functional analyzes of some of the miRNAs detected in microarray studies that another group had previously performed. And as a result of this research, we obtained very important data.

In this work, miRNAs related to human laryngeal squamous cell carcinoma was investigated and, 5 different miRNAs were selected from miRNAs, which was found significant in earlier studies. These miRNAs are mir-26b, mir200c-3p, mir-203, mir363-3p, and mir-1825. These miRNAs were studied in Hep-2 cell, is a squamous cell carcinoma cell line. With trisol, RNA isolation was performed for this. Subsequent cDNA synthesis and qRT-PCR studies were performed. In order to transfect these miRNAs, cell culture studies were performed by using Hep-2 cells. After passing through many passages, cells that have reached enough confulence have become ready for transfection. Transfection, proliferation, migration and invasion assays were performed respectively. According to the proliferation findings, mir-26b, mir200c-3p and mir-1825 did not show a significant decrease or increase on proliferation to Hep-2 cells.

For this reason they were not included in further functional analyzes. mir-203 and mir-363-3p a noticeable reduction in the proliferation abilities of Hep-2 cells was determined. These findings indicate that mir1825, mir-200c-3p and mir-26b have no effect on the tumorigenic process, but mir-203 and mir-363-3p may have a great effect. The result of this meaningful outcome continued at the advanced functional stages.

The graphics obtained in the migration and invasive assays made within the scope of our study revealed that we were on the right track. Significant reductions in mir-203 and mir-363 transfected Hep-2 cells were detected compared to the migration and invasion capacities of normal Hep-2 cells, according to the results obtained. These results led to the finding that mir203 and mir363 were tumor suppressor and that cancer cells had a negative effect on their ability.

The effect of miRNAs on the ability of tumor cells to colonize was also investigated. According to the results, it was determined that in the miR-203 and miR-363-3p transfected cells, the control group significantly decreased their ability to form colonies as compared to hep-2 cells. Quantitative statistical analyzes supported these results.

Based on the results of the study, mir-363-3p and mir-203 have been proven to be tumor-suppressing. These miRNAs have been found to significantly reduce cell proliferation, migration, invasion and colony formation from their natural ability of tumor cells.We have proven that our hypothesis is correct in the end result of our work. We found positive effects on squamose cell laryngeal carcinoma detected in miR-203 and miR-363-3p among 5 different miRNAs. We have determined that mir1825, mir-200c-3p and mir-26b have no effect on squamose cell laryngeal cancer. It is understood that mir-203 and mir-363-3p can easily be used as candidate for diagnostic markers according to the data obtained in this study. This study will lead the work to be done in pharmaceutical fields. It will provide a step for the progress of the disease in the diagnosis treatment. This study might shed a light further experiments in order to understand this cancer mechanisim.

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IN VITRO CHARACTERIZATION OF MICRORNAS SPECIFIC TO CANCER STEM CELL IN LARYNX CANCER

ORIJINALLIK RAPORU

%26	%22	%19	%12
BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	Submitted to Istanbul University Öğrenci Ödevi	%1
2	Submitted to Postgraduate Institute of Medicine Öğrenci Ödevi	%1
3	emedicine.medscape.com İnternet Kaynağı	%1
4	link.springer.com İnternet Kaynağı	%1
5	Pedro Pinheiro. "The Role of MIR-375 in Prostate Carcinogenesis", Repositório Aberto da Universidade do Porto, 2014. Yayın	%1
6	R. Bryan Bell, Rui P. Fernandes, Peter E. Andersen. "Introduction", Elsevier BV, 2018 Yayın	%1
7	www.ncbi.nlm.nih.gov İnternet Kaynağı	%1

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	Position	Company	Time (Year-Year)
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2.			

Academic Publications

Name	Publication Year
Differential expression of kallikreins as prognostic markers in recurrent prostate cancer.	Poster precentation in ASHG-2017

Foreign Languages	**Writing**	**Speaking**	**Reading**	**YDS**	**Other**
English	Intermediate	Intermediate	Advance	71.25	-

	Analytic	**Quantitative**	**Verbal**
ALES	77.08	77.23	68.30

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