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ORIGINAL ARTICLE

Evaluation of *UCA1/miR-138/CDK6* Network in the Patients with Laryngeal Squamous Cell Carcinoma

Laringeal Skuamöz Hücreli Karsinomlu Hastalarda *UCA1/miR-138/CDK6* Ağının Değerlendirilmesi

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ABSTRACT

Objective: Noncoding RNAs (ncRNAs) have the potential to be diagnostic and therapeutic targets, in many cancer types. miRNA mRNA ceRNA (competing endogenous RNA) network in cancer has been an interesting topic in the recent research.We aimed to investigate the relationship of The urothelial cancer-associated one gene *(UCA1)/miR-138/CDK6* network with laryngeal squamous cell carcinoma (LSCC).
Material and Methods: We studied the samples of adjacent normal tissue from patients diagnosed with LSCC in addition to the samples of malignant tissue. Following the RNA isolation of the samples, *UCA1, miR-138, and CDK6* expression analysis was performed using the quantitative real-time PCR (qRT-PCR) method.
Results: Expressions of cancerous tissue samples and adjacent normal tissue samples as controls were compared. *CDK6 and UCA1* levels were increased and *miR-138* levels were found to be decreased in cancer tissues but not statistically significant.
Conclusion: To the best of our knowledge there is no study to provide an expression profile of the *UCA1/CDK6/miR-138* network for LSCC patients. Although we could not obtain statistically significant results, our results were similar with the *UCA1, miR-138, and CDK6* axis literature. Further studies with larger patient samples and LSCC cell lines may confirm the function of this axis, which might be a diagnostic and therapeutic target for LSCC.

Keywords: Long non-coding RNA, lncRNA, Laryngeal cancer, miRNA, *UCA1, miR138, CDK6*

ÖZ

Amaç: Kodlamayan RNA'lar (ncRNA'lar), birçok kanser türünde tanı ve tedavi hedefi olma potansiyeline sahiptir. miRNA – mRNA ceRNA (yarışmalı endojen RNA) ağı son yıllarda üzerinde çalışılan ilginç bir konu olmuştur. Bu çalışmayla *UCA1/miR-138/CDK6* ağının laringeal skuamöz hücreli karsinom (LSCC) ile ilişkisini araştırmayı amaçladık.
Gereç ve Yöntem: LSCC tanısı alan hastaların kanserli dokuları ve kontrol olarak da komşu normal doku örneklerinden çalışma yapıldı. Örneklerden RNA izolasyonu yapıldıktan sonra kantitatif gerçek zamanlı PCR (qRT-PCR) yöntemi ile *UCA1, miR-138 ve CDK6* ekspresyonları değerlendirildi.
Bulgular: Kanserli ve kontrol doku örneklerinin ekspresyonları karşılaştırıldı. Kanser dokularında, istatistiksel olarak anlamlı olmamakla birlikte *CDK6 ve UCA1* ekspresyonunun arttığı, *miR-138* ekspresyonun ise azaldığı belirlendi.
Sonuç: Çalışmamız, bildiğimiz kadarıyla LSCC hastalarında *UCA1/CDK6/miR-138* ağı ekspresyon profilinin çalışıldığı ilk çalışmadır. İstatistiksel olarak anlamlı sonuçlar elde edilememiş olmakla birlikte, sonuçlarımız *UCA1, miR-138 ve CDK6* ağ çalışma literatürüyle benzerdi. Örnek sayısının daha fazla olduğu çalışmalar ve LSCC hücre dizileri ile yapılacak ileri çalışmalar, LSCC için tanı ve tedavi hedefi olabilecek bu yolağın fonksiyonlarını daha da netleştirecektir.

Anahtar Kelimeler: Uzun kodlamayan RNA, lncRNA, Laringeal kanser, miRNA, *UCA1, miR138, CDK6*

Introduction

Laryngeal cancer is a common type of head and neck cancers and laryngeal squamous cell carcinoma (LSCC) is the most common type of laryngeal cancer with a rate of 90% (1). The long-term prognosis of LSCC patients is considered poor because of frequent metastases and recurrences (2). Therefore, it is essential to identify potential therapeutic targets. Although substantial breakthroughs have been achieved in therapeutic techniques such as surgery, chemotherapy, and radiotherapy, the long-term prognosis of LSCC patients is not adequate due to frequent metastases and recurrences (3). Investigating regulatory mechanisms in LSCC development and progression will contribute to developing effective therapeutic strategies for LSCC. From this perspective, selecting patients who will benefit from a particular treatment and identifying new biomarkers that predict the clinical outcome will be essential (4). Studies based on DNA sequencing ((5–7), determination of the expression level of RNAs (8,9), and detection of the amounts of translational proteins (10,11) have an important place in the early and differential diagnosis, prognosis and etiopathogenesis of diseases and in developing appropriate treatment strategies.

In the human genome, non-coding RNA genes form functional RNA molecules without coding for proteins and act as regulators in processes that have critical

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roles in the cell (12). It is known that non-coding RNAs (ncRNAs) have a role in controlling signaling pathways (13). Among these ncRNAs,long noncoding RNAs(lncRNAs) are the main focus of interest. lncRNAs are classified as non-protein-coding RNA transcripts longer than 200 nucleotides.

In recent years, there has been a greater focus on the role that lncRNAs and miRNAs play in the etiology of cancer. (13). In general, lncRNAs change gene expression levels and play a regulatory role in transcription and post-transcriptional processes, particularly in chromatin remodeling (14). In the current literature, it has been reported that lncRNAs play a role in tumourigenesis and tumour progression and show metastatic properties in various cancer conditions (15).

MiRNAs, are non-coding RNAs which consist of about 19–25 nucleotides and bind to 3′ untranslated region (3′ UTR) of target genes. Abnormal expression of miRNAs contribute the cancer process. Oncogenic or tumour suppressor miRNAs have been revealed in various cancers (16). According to the ceRNA hypothesis,described by Salmena et al., lncRNAs can act as ceRNAs by competing with miRNAs and preventing them from binding to specific binding sites on mRNA, thereby regulating target genes posttranscriptionally (17). Various studies have shown that ceRNA network plays a vital role in tumour development (18).

In this study, we aimed to investigate the expression levels and interrelationship of the UCA1-miR138-CDK6(lncRNA-miRNA-mRNA) ceRNA network in the patients with LSCC.

Materials And Methods

Selecting Patients and Keeping Records

This study was planned as a case-control study. Ethics committee approval was obtained from Atatürk University Faculty of Medicine Research Ethics Committee (approval number: B.30.2.ATA.0.01.00/60).. Thirty patients between the ages 18-70 with LSCC diagnosis were included in the current study. Written informed consent was obtained from all participants. From March 2020 to March 2021, laryngeal tissue samples of individuals with the diagnosis of LSCC in the ENT (Ear-Nose-Throat) clinic were surgically removed(with confirmed frozen biopsy), and thirty pairs of LSCC tissue and matching adjacent normal tissue samples were collected. None of the patients received chemotherapy, radiotherapy or biotherapy before surgery. Diagnosis of the patients were confirmed by pathological analysis. All samples were frozen in liquid nitrogen within 5 minutes after resection and then stored at -80°C until further use for RNA analysis.

After considering the inclusion and exclusion criteria, *UCA1, miR-138, and CDK6* expression analysis was performed after total RNA isolation from tissues or cells, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR). Several control genes were included in the study to ensure normalization. Control genes *GAPDHP61* for lncRNA, *GAPDH* for mRNA, and RNU6 for miRNA were selected.

Expression Analysis of RNAs

Bioinformatics tools such as miRcode and TargetScan developed to identify competing triplets associated with lncRNA were used to predict miRNA-lncRNA interactions. MiRNA-mRNA interactions were obtained from two high-quality databases, miRDB and mirTarBase. LncRNA-mRNA pairs sharing a miRNA, the *UCA1/miR-138/CDK6* network was constructed as a candidate for lncRNA-miRNA-mRNA ceRNA axis.

Analysis of Data

During the PCR reaction, as the amplification process took place, the released fluorescence given by the SYBER Green dye was recorded by the Real-Time PCR, and the Ct values of each sample according to the initial concentration were automatically calculated by the device. Ct values and Ct curve graphs of 30 pairs of samples accepted as patientand control were analyzed and recorded in the Rotor-Gene Software program.LncRNA, mRNA, and miRNA expression changes were calculated by the comparative Ct method known as the $2^{-\Delta\Delta Ct}$ (Livak) method. Fold change=$2^{-\Delta\Delta Ct}$ is the normalized miRNA expression in each test sample divided the normalized miRNA expression in the control sample. Average Ct values for each gene of the groups of patients and controls were calculated. Average ΔCt and $\Delta\Delta Ct$ values were calculated. ΔCt = Ct Target Gene – Ct Reference Gene. $\Delta\Delta Ct$ = Group of the patients ΔCt – Group of the controls ΔCt). Within the scope of the study, the data were summarized using descriptive statistics. For this purpose, mean values were calculated for RNA expression levels. Student's T-test was used to compare two independent groups in terms of customarily distributed numerical variables. A value of $p<0.05$ was considered statistically significant.

Results

Expression Analysis Findings

The expression levels of *UCA1, CDK6, miR-138* genes were determined in the patient and control groups by normalizing with the *GAPDH, GAPDHP61 and RNU6* reference genes. Ct values of 30 pairs of samples accepted as case and control were analyzed (Tab.1)

Table 1. Mean Ct and Δ Ct values detected for *UCA1, CDK6, miR-138, GAPDH, GAPDHP61 and RNU6*

	Average Ct values		Average Δ Ct values (Ct (target RNA)- Ct (reference RNA))	
	Patient group	Control group	Patient group	Control group
UCA1	26,43	27,06	6,95	7,29
CDK6	22,26	23,56	3,59	4,80
miR-138	20,12	20,87	0,88	-1,95
GAPDHP61	14,98	15,97	0	0
GAPDH	20,11	19,76	0	0
RNU6	19,23	22,00	0	0

UCA1, miR-138, and CDK6 expression levels in LSSC and adjacent normal tissues as controls were examined and fold changes were determined (Tab.2)

Table 2: 'Fold Change' of *UCA1, CDK6*, miR-138, compared to controls and p values

	Fold Change	p - values
UCA1	1,27	0,35
CDK6	2,32	0,08
miR-138	0,28	0,25

Fold change: $2^{-\Delta\Delta Ct}$ (ΔΔCt = Patient Group ΔCt – Control Group ΔCt)

The fold change of *CDK6* was increased 2.32 times in laryngeal cancer compared to control (p< 0.08). It was not found to be statistically significant (Figure 1).

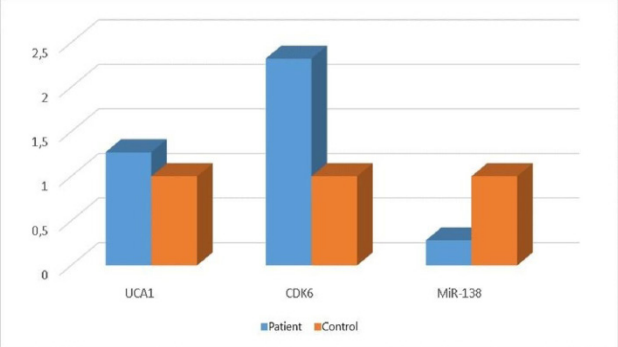

Figure 1. Fold change values of patient and control groups.

UCA1 was expressed 1.27 times higher in LSCC tissues compared to controls, but it was not found to be statistically significant. However, a 0.28-fold decrease in *miR-138* RNA levels was detected in LSCC tissues compared to controls. The difference in miRNA expression levels between the two groups was not statistically significant. (Figure 1).

Discussion

UCA1, an oncogenic lncRNA, was initially identified in bladder cancer tissues. It has been demonstrated that *UCA1* is upregulated and has carcinogenic effects in various cancers (19). In a study by Sun et al., *UCA1* was significantly upregulated in most LSCC patients, and it enhanced the ability of LSCC cells to proliferate, migrate, and invade by activating the Wnt/ β-catenin signaling pathway in tumour tissues compared to the adjecenthealthy tissues (20). It was found that overexpression of *UCA1* in lung cancer increased cancer cells' proliferation, migration, and invasion abilities reversing the tumour suppressor effect of *miR-138 and miR-193* in a study on the *UCA-1, miR-138, miR-193, and CDK6* axis. In the same study, a negative correlation was shown between *miR-138 and miR-193 and UCA1* expressions. These findings supported that *UCA1* might control *CDK6* expression by targeting *miR-138 and miR-193*, which are both targets of *UCA1 and CDK6* (21). We aimed to study this axis, which has been studied in lung cancer, in LSCC. Although we could not obtain statistically significant results, our results were similar with this data; *UCA1 and CDK6* expressions were up regulated while *miR-138* was down regulated in the *UCA-1, miR-138, and CDK6* axis. In another study on *UCA1/miR-185-5p/ HOXA13* axis on LSCC tissues and cell lines, UCA1 expression was upregulated, and UCA1 regulated the proliferation and migration of the cells via the *miR-185-5p/HOXA13* axis (22).

MiR-138 is highly conserved among vertebrates and has been shown to play a role in organogenesis (16). Dysfunction of *MiR-138* has been observed in various tumour types. *MiR-138* has been defined as a tumour suppressor in cancers by targeting various oncogenes. In a study by Wang et al., *miR-138* was selected as a candidate miRNA in the *TRPM2-AS /miR-138/SOX4* axis (23). They found that *TRPM2-AS* sponged *miR-138* to eliminate its repression on *SOX4* function in LSCC cells and *miR-138* was down regulated (23). It has been shown that *miR-138,* is down regulated in cancer cells, targets histone methyltransferase *EZH2*, and reduces the metastasis of cancer cells (24). *EZH2 and miR-138* relationship has also been documented in several other forms of cancer such as; clear cell renal cell cancer, nonsmall cell lung cancer, osteosarcoma and glioblastoma (25) . In another study, the decrease of *miR-138* expression in LSCC cell lines and the suppressive role of *miR-138* in LSCC cell proliferation by inhibiting *EZH2* expression and the *PI3K/AKT* pathway has been reported. As a result, it has been postulated that the

miR-138/EZH2 axis may be a promising therapeutic target for LSCC (26) . Gao et al. reported that *miR-138* was downregulated in LSCC tissues compared to matched normal laryngeal tissue. *ZEB2*, the target of *miR-138*, was conversely upregulated. They suggested *miR-138* as a potential therapeutic target for *ZEB2*-induced LSCC (27). In our study, *miR-138* was found downregulated in LSCC samples in accordance with the literature, but it was not found to be statistically significant.

CDK6 is the main regulator of the G1/S cell cycle transition. *CDK6* expression is high in head and neck squamous cell carcinoma and is associated with tumour progression. *CDK6* plays critical roles in cell proliferation, cycling, differentiation, and metastasis. *CDK6* expression has been shown to be upregulated and functions as an oncogene in LSCC(28) . In a study of *CDKN2B-AS1/mir-497/CDK6* axis in LSCC, it was reported that the lncRNA; *CDKN2B-AS1* targets *miR-497/CDK6*. They found that *CDK6 and CDKN2B-AS1* was up regulated and *miR-497* was downregulated in LSCC tissues. With these results, they suggested that *CDKN2B-AS1* is a *miR-497* sponge in LSCC cells and thus upregulates *CDK6* expression (29) . In our study, *CDK6* expression was increased in LSCC tissues compared to adjacent healthy tissues, in agreement with the previously published reports.

Biomarkers are widely used as powerful tools for patient diagnosis, disease staging and monitoring, and predicting clinical prognosis (28). The potential of ceRNAs as biomarkers is further enhanced by the fact that ncRNAs are found in body fluids such as blood, urine, or in the extracellular vesicles, including exosomes. The ceRNA network have been characterized in various cancers in recent years(14,18). The discovery of these interactions has offered a new perspective on cancer diagnosis, prognosis and therapy. In our study, the *UCA1/miR-138/CDK6* axis, one of the ceRNA networks with the potential to be a biomarker in cancer, was studied in LSCC tissues.

The ceRNA network on cancer diagnosis, prognosis and therapy is an intriguing issue. To the best of our knowledge there is no study to provide an expression profile of the *UCA1-CDK6-miR-138* ceRNA network for LSCC patients. Although we could not obtain statistically significant results, our results were similar with the *UCA-1, miR-138, and CDK6* axis literature. Sample number is a limitation of our study. Further studies with larger patient samples and LSCC cell lines may confirm the function of this axis, which might be a diagnostic and therapeutic target for LSCC.

Statements and Declarations

Ethics Committee Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Atatürk University (B.30.2.ATA.0.01.00/60). Written informed consent was obtained from all participants.

Conflicts of interest

The authors declare no conflict of interest.

Financial Disclosure

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Authors' contributions

Concept and design: NC, CYK, AT, literature search: NC, CYK, data acquisition and analysis: NC, CYK, AT, manuscript preparation: NC, CYK, manuscript editing and manuscript review: NC, CYK, AT. All authors contributed to manuscript drafting and approved the final version.

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