

## Investigation of SKOR2 Gene Expression in Patients with Lung Cancer Using Real-time PCR

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### Authors' contributions

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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### ABSTRACT

**Background and Aims:** Lung cancer includes small cell lung cancer and non-small cell lung cancer (NSCLCs) types. NSCLCs are investigated for various mutations, which is available can be treated with targeted new molecular therapies. The aim of this study was to evaluate the expression of SKOR2 gene, disease severity, and evaluation of NSCLC and its subtypes of cancer patients among patients with lung cancer in Tehran hospitals using Real-Time PCR.

**Materials and Methods:** A total of 35 clinical samples were collected from patients with NSCLC-derived lung cancer from three hospitals in Tehran. The range of patients varied from 37 to 80 years. The disease grade ranges in the patients in this study were varied and 22 different grades were observed. In order to evaluate the SKOR2 gene after extraction of RNA and cDNA synthesis, the gene expression was evaluated using Real-Time PCR.

**Results:** Of the 22 observed grades, the highest grade IIIa grade was observed in 6 patients (17.1%). 74% of adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade IIIa. Patients with T3 stage included 4 samples, 2 of which were adenocarcinoma and

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2 were SCC and their age ranged from 55 to 62 years old. Three patients were in the T1 category, and 100% of them had adenocarcinoma. The results showed that expression of SKOR2 5.47 fold in lung cancer patients with NSCLC was more than normal patients.

**Conclusion:** According to the results, expression of the SKOR2 gene in the tissues of individuals with lung cancer was increased compared to normal individuals. The expression of this gene in patient suggests the possibility of involvement of this agent in the progression of the disease. According to the results, it is recommended to use an evaluation of the expression of other biomarkers in lung cancer to help with this and to increase the accuracy of screening tests for lung cancer.

*Keywords: Lung cancer; NSCLC; Real-time PCR; SKOR2.*

## 1. INTRODUCTION

Lung cancer is histologically divided into small cell lung cancer and NSCLC types. The most common symptoms of lung cancer include a cough, sore throat, hemoptysis and systemic symptoms, such as weight loss and anorexia. If the risk of lung cancer is high, a diagnostic evaluation is essential. Diagnostic assessment has three synchronous stages (tissue diagnosis, staging, and functional evaluation), all of which affect planning and prognosis. Small cell noncancerous cancer cells (NSCLCs) are tested for various mutations, which is available can be treated with new targeted molecular therapies. Tobacco use accounts for 80% to 90% of all lung cancer cases. Exposure to cigarette smoke is also an important risk factor. Risk factors are usually dose-dependent and time-consuming and many carcinogens are involved in process tobacco smoking [1-3].

NSCLC is divided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC is sometimes weak and can only be detected by immuno histochemical and molecular testing. The problem is when only a small amount of tissue is available for testing. The optimal treatment depends on the complete evolution of phenotype and tumor genotype [4].

The SKOR2 gene, also known as CORL2, CH18515, and FUSSEL18, was firstly identified in silico investigations for new homologous genes belonging to the family Ski/Sno of transcriptional co-repressors [5]. It is also known as FUSSEL18 (the Smad suppressor agent on chromosome 18) and is highly homologous to the LBX1 co-repressor and is referred to as FUSSEL15 [6,7]. Although it has been shown that Corl1 is a compressor for LBX1, the role of SKOR2 is not well defined. Similarly, Ski/Sno, SKOR2 has the second two domains in the N-terminal region and the second Dachshund

homology domain (DHD) [8] and a SAND adjacent domain necessary for the Ski/Sno interaction with Smad4 [9].

It has been shown that Ski/Sno is a negative regulator of the bone marrow protein (BMP) signaling pathways and TGF-B growth factor by binding to the Smad protein [10]. Ski and its dependent SnoN are recognized as oncogenes and are capable of leading to high overexpression of embryonic fibroblasts genes. While the high expression of Ski and SnoN in many human tissues and cells has also been reported to have an oncogenic activity, evidence suggests that potential antitumor activity has been proposed for both of these genes. In addition, Ski and SnoN play a role in regulating cellular differentiation, particularly in neuronal and muscular cells [11].

NSCLC is one of the causes of cancer death in worldwide; therefore, improvement in diagnosis and treatment is necessary [1]. Lung cancer grows in four stages, and it is difficult to detect it early in the stage of cancer and is commonly detected at stages three and four. In the first stage, it is located in the lungs, and in the second stage, it spreads to the lymph nodes. In the third stage, it extends to the chest and disseminates to other parts of the body at the final stage [1,12].

The aim of this study was to evaluate the expression of SKOR2 gene, disease severity, and evaluation of NSCLC and its subtypes among patients with lung cancer in Tehran hospitals using Real-Time PCR.

## 2. MATERIALS AND METHODS

### 2.1 Clinical Samples

In this study, 35 clinical samples from patients with NSCLC-derived lung cancer were collected

from three hospitals in Tehran including Khatam hospital, Atiyah hospital and Masih Hospital. The majority of samples was from Masih hospital (71.4%), followed by 22.8% from Atiyah Hospital, and 5.7% from Khatam Hospital collected in 2017, among which 20% of the patients were women and 80% of them were men. The mean age of patients varied from 37 to 80 years.

### 2.1.1 Patients' clinical data

The disease grade gradients in the patients in this study varied and 22 different grades were observed. Grades obtained included IB, IIB, IIA, III, IIIa, T1aN0MX, PT4N0MX, T1bN) MX, T1N1MX, T2aN2MX, T2aN0, T2, T2AN2MX, T2BN0MX, T2N0, T2aN0M0B1, T2BN1T2N1, T3N0MX-IIB, T3N1MX, T3N0MX and T3N2MX grades. Six patients had an unknown stage of their disease.

All of the three adenocarcinoma, SCC, and adenosquamous subtypes and were observed in the study of subtypes of the disease. Sixteen subtypes were adenocarcinoma, 12 subtypes were also identified as SCC and 1 adenosquamous subtype was detected. The only adenosquamous subtype was belonged to the T2BN0MX Grade and detected in the Masih Hospital.

## 2.2 Real-Time PCR

### 2.2.1 RNA extraction

Firstly, approximately 50 to 100 milligrams of tissue samples were separated with a scalpel and placed it on a plate. Next, the tissue samples were transferred into an RNase, DNase free microtube and 1000  $\mu$ l TRIZOLE was added to the mixture and after a vortex, the cells were completely lysed and no visible particles remained in the solution and thus we obtained a homogeneous solution. Then, 200  $\mu$ l of chloroform was added to the mixture which contained the TRIZOLE to isolate each of phases and by inverting for several times. At the next stage, each sample was centrifuged at 13,000 rpm for 10 minutes at 4°C.

After centrifugation, three phases were observed. The pink color is below exhibiting TRIZOLE, a white precipitated layer which contained blood proteins and the blue anhydrous supernatant phase which contained RNA were included. the supernatant containing RNA was slowly and accurately taken without uptake of the below layer using an RNase and DNase free tip in a

volume of 400  $\mu$ l, and placed to a new 1.5 RNase and DNase free microtubes, and thereafter to the same amount (400  $\mu$ l) isopropanol was added to it and subsequently incubated for 10 minutes at room temperature.

In the next step, centrifugation was conducted for 5 minutes at 13000 rpm and 4°C. The supernatant phase was then completely removed to allow only RNA deposition in the microtubule. RNA remained was next added to a 1000  $\mu$ l of 75% ethanol and washed. The solutions were then re-centrifuged for 5 minutes at approximately 10,000 rpm at 4°C and thereafter, ethanol was completely removed.

After that, the microtubes were put on ice for 10 min to completely dry the RNA sediment. Based on the volume of the obtained RNA, the amount of 30-50  $\mu$ l DEPC water was added and the RNA sedimentation was completely dissolved by pipetting. Finally, with reading the OD, 3 $\mu$ l of extracted RNA was taken to evaluate its concentration using NanoDrop apparatus measuring and the RNA was immediately transferred to -70C conditions.

### 2.2.2 cDNA synthesis

In the first step, the Real-Time PCR method of the RNA molecule (gene transcription) is used as a template for the synthesis of the cDNA molecule, for this aim, forward and reverse primers, an RT enzyme Primescript (Takara) and oligonucleotides and oligomer dioxymidine (Oligo-dt) are needed in order to complete the polymerization reaction. Next, in a thermal cycler, as in the PCR, the cDNA molecule extends logarithmically. For quality control of extracted total RNA samples, this RNA was tested using spectrophotometry and also on the 1% agarose gel.

## 2.3 Real-time PCR Conditions

There are several methods for implementing the real-time PCR such as probes and intercalating dyes. The SYBR Green fluorescence dye has less and, of course, more susceptibility when attached to the target sequence than a variety of probes. The free molecules of this intercalation dye integrate into two strands of DNA synthesized per cycle and emit fluorescent signals.

The threshold fluorescence is a point at which a marked change in the intensity of the

**Table 1. Primers used the expression of the SKOR2 gene**

Primer TM	Sequences 5'-3'	Primer name
60.5	5'-CTG CGG CAT GAT CAC CAA AC-3'	SKOR2-F
60.5	5'-ACT GGT GTG ACA CGT CGA AG -3'	SKOR2-R

**Table 2. Materials used in the reaction of the real-time PCR for the SKOR2 gene**

Materials	Volume ( $\mu$ L) in the reaction
RealQ Plus 2x Master Mix Green - Amplicon	7.5
Primer Mix Forward and Reverse (3 $\mu$ M each)	1
cDNA (10 ng/ $\mu$ L)	1
Distilled Deionized Water	5.5
Final volume	15

**Table 3. Temperature and time used for real-time PCR for the SKOR2 gene**

-	Temperature $^{\circ}$ C	Time	Repeat
Hot Start Activation	95	15 min	-
Denaturation	95	15 S	40
Annealing and Extension	60	1 min	-

fluorescence signal occurs over the background fluorescence. The linear threshold line is automatically plotted by the device and specifies the boundary between background fluorescence signals and meaningful fluorescence signals associated with the extension. The threshold line or level is automatically plotted by the device 10 times the standard deviation of the baseline in the progressive phase of the expansion curves. The relationship between the two variables is the number of cycles and the number of products produced as an exponential equation, and its logarithm is a linear equation. In other words, for a whole region with a progressive relation there, one can select a point and draw a line from the threshold. In contrast, the cycle threshold is a cycle in which each reaction starts its progressive amplification; and a significant increase in the fluorescence signal is detected, and the PCR curve in this cycle disconnects the threshold line. Determining the threshold cycle in the progressive increasing phase of extension is a basis for quantitative analysis in the Real-Time PCR system.

Primers and materials applied with the Real-Time PCR thermal conditions are exhibited in Tables 1, 2 and 3.

## 2.4 Ethical Approval and Consent

The study was approved by the Institutional Review Board of Central Tehran Branch, Islamic Azad University, Tehran, Iran and performed in accordance with the principles of the Declaration of Helsinki and each subject signed an informed

written consent before participating to the study. All procedures were approved by the relevant ethics committees, and written informed consent was obtained from all participants.

## 3. RESULTS

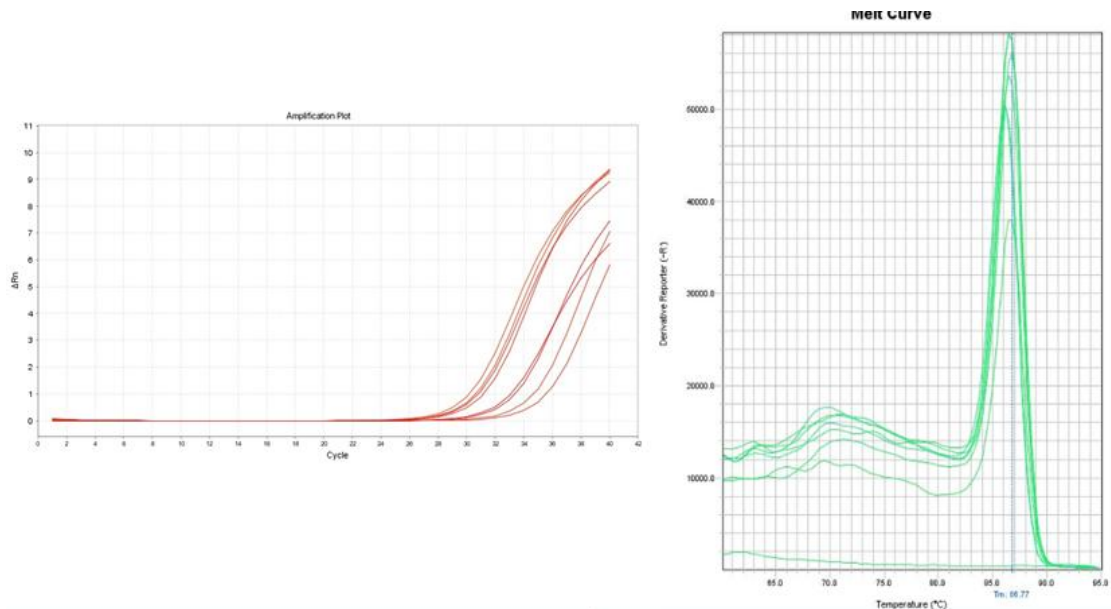
Patient specimens and disease gradients: The frequency of 22 grades of the disease observed in patients in this study is shown in Fig. 1. The highest grade which was IIIa included 6 patients (17.1%).

An interesting finding was that 74% of adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade IIIa.

Patients with the T3 stage of the disease included 4 of them, 2 of which had adenocarcinoma and 2 were SCC and their age ranged from 55 to 62 years old. Three patients were in the T1 category, and 100% of them had adenocarcinoma. Patients placing in the IIA and IIB categories belonged to the SCC subclass, and all three patients were from Atiyah Hospital.

Only one IB case was determined in the patients, which was also classified under the SCC subclass.

Independent t-test demonstrated that the average age of the patients with a subtype of adenoma was 56.7 years and the SCT subtype was 60.7 years, and this difference was not statistically significant between the two groups ( $p = 0.202$ ).



**Fig. 1. Linear and logarithmic diagrams of Real-Time PCR amplification and temperature curve for the SKOR2 gene**

The linear and logarithmic graphs of real-time PCR amplification and thermal curve for the SKOR2 gene are shown in Fig. 2. The results revealed that expression of SKOR2 was increased 5.47 fold in lung cancer patients with NSCLC compared to those having normal status.

#### 4. DISCUSSION

Lung cancer is one of the most frequently diagnosed cancers and the leading cause of death due to cancer worldwide. An NSCLC, a heterogeneous tumor class, accounts for about 85% of all new lung cancer cases. Smoking is still a major cause of the disease, but exposure to radon and air pollution plays an important role in this regard. Most patients are diagnosed with inadequate screening and clinical symptoms. As a result, patients have a very poor and unclear prognosis [13,14].

NSCLC, which includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for approximately 85% of new cases of lung cancer. Moreover, SCLC includes 15% of the rest cases [15,16].

It should be noted that from the practical viewpoint of the laboratory, the above results are when tumor samples are used instead of cancerous cells in an attempt to evaluate the

expression of any gene for cancer. There are cases whereby these genes can typically be misleading and confused by a cell type among the many who express the release of tumors. This possibility also refers to unidentified genes that may be unwittingly generated by secret messages from the tumor.

Some screening tests have proven to play an important role in the outcome of the illness and its treatment. Among these tests, tumor biomarkers that are found to respond to the presence or progression of cancer from the body or the tumor tissue and found to be abundant in blood, urine and tumor tissue compared to those healthy people are suitable criteria for early diagnosis of the disease. [17,18]. Increasing the level of these biomarkers, indicating the presence of cancer and reducing their amount during treatment is an indication of inhibiting tumor growth in the patient [19]. Although today these markers are mainly used to diagnose various cancers, the marker for malignant cancers has not yet been identified [20]. Among various types of tumor markers, mRNA biomarkers appear to be more appropriate than others, since the very little amount of this marker can be searched and evaluated using the Real-Time PCR technique, which is a method with a high sensitivity and specificity, and that's why the biomarker from the same group was named SKOR2.

## 5. CONCLUSION

Molecular techniques, including PCR, are more rapid, accurate, proper and cheaper and easier than microscopic techniques for tracking tumors. According to the results, expression of the SKOR2 gene in the tissues of individuals with lung cancer was increased compared to normal individuals. The lack of expression of this gene in normal specimens and its expression in patient samples suggests the possibility of involvement of this agent in the progression of the disease and it seems that the increase in the expression of SKOR2 in the development of disease and thereby the role of this factor in therapy purposes seems essential. According to the results, it is recommended to use a review of the expression of other biomarkers in lung cancer to help with this and to increase the accuracy of screening tests for lung cancer.

## CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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