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# Detecting mRNA Expression of *LUNX* Gene by RT-PCR in Lung Cancer Specimens of Iraqi Patients

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**Abstract:** This study was conducted to evaluate the clinical significance of mRNA expression of lung-specific X protein (*LUNX*) in specimens obtained from Iraqi patients. In the present study, whole blood was isolated from 140 individuals distributed into four groups as follows; Group 1: 40 Samples from smoker patients affected by lung cancer, Group 2: 40 Samples from non-smoker patients affected by lung cancer, Group 3: 30 Samples from smokers apparently healthy individuals and Group 4: 30 Samples from nonsmokers apparently healthy individuals. The messenger RNA (mRNA) expression levels of *LUNX* in the peripheral blood were analyzed using reverse transcription‐polymerase chain reaction (RT‐PCR). The expressions of *LUNX* mRNA in these groups were 11.0 folds of that in group 1 compared with group 4, 10 folds of gene Expression in group 2 compared with group 4 and 3.0 folds of gene expression in group 3 compared with group 4, using *GAPDH* as Housekeeping Gene. Statistical results showed that there was a high correlation between the *LUNX* gene expression and lung cancer; where the correlation coefficient was 0.879 and a low correlation between *LUNX* gene expression and smoking; where the correlation coefficient was 0.446. The results indicate that the mRNA expression of *LUNX* could yield a first picture to the early detection of lung cancer in the peripheral blood and thus is of significant clinical value for the diagnosis of lung cancer.

**Key words:** mRNA, LUNX Gene, RT-PCR, Lung Cancer, Iraq.

## Introduction

Cancer is one of the most important health problems of the current era and also a leading cause of death among populations . Lung cancer is the leading cause of cancer-related deaths in both men and women in many countries throughout the world. The majority of cases (~60%) are diagnosed after the disease has spread to distant areas and the 5-year survival rate for those with advanced disease is only around 4%. However, if detected early, when the tumor is still localized, the 5-year survival rate is much higher at 55%-75% with many of these being cured.

The majority of cases of lung cancer are due to long-term tobacco smoking; however, other cases could occur in people who have never smoked. It has been postulated that those cases are often caused by a combination of genetic factors and exposure to radon gas, asbestos, and or other forms of air pollution (1, 2).

Lung-specific X (LUNX; also known as BPIFA1, PLUNC, and SPLUNC1) is a member of the palate,

lung, and nasal epithelium clone (PLUNC) protein family and is the human homolog of murine plunk, and like the mouse gene, is specifically expressed in the upper airways and nasopharyngeal regions (3). It is a lung-specific gene that is highly expressed in the NSCLC (non-small cell lung cancer) type of lung cancer. Experimental evidence suggests that *LUNX* may be considered as a diagnostic biomarker for lung cancer and be able to determine micro-metastases in lymph nodes of NSCLC patients and peripheral blood (4). Sequential analysis shows that *LUNX* may play a role in intrinsic immunity. The exact morphologic function of this gene is unknown, but it has been reported that it plays a role in inflammatory responses to upper respiratory tract stimulation. In addition, it has particular value for improving the prognosis of patients (5) (6).

Determination of the expression level of some genes in cancer patients by real-time RT-PCR can therefore be beneficial in its earlier stages (7,8,9). At

present, reverse transcription -polymerase chain reaction is used for the detection of RNA (mRNA) expression levels and to quantify the expression of tumor markers and genes associated with tumors in the peripheral blood. This study was conducted to evaluate the clinical significance of mRNA expression of lung-specific X protein (*LUNX*) in specimens obtained from Iraqi patients. mRNA expression levels lung-specific X protein (*LUNX*) were assessed and the clinical significance of the mRNA levels was evaluated.

## Materials and Methods

This study was conducted spanning the time from August 2015 to May 2016. All the study experiments were performed at the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / University of Baghdad and the National Cancer Research Center (NCRC) of University of Baghdad.

### Study groups

The total number of participants in the study was 140 individuals grouped as follows:

**Group 1:** Eighty patients diagnosed with lung cancer in both Al–Amel National Hospital and the Oncology Teaching Hospital belonging to the Medical City in Baghdad. Patients from both genders at different age groups were included. Their clinical information were obtained from their hospital files and case-sheet records; divided into-: **1.** Forty samples from smoker patients affected by lung cancer. **2.** Forty samples from non-smoker patients affected by lung cancer.

**Group2:** Sixty apparently healthy individuals of both sexes at different ages. They were subdivided into two groups -: **3.** Thirty samples of smokers apparently healthy individuals. **4.** Thirty samples of non-smokers apparently healthy individuals .

### Blood Sampling:

From each participant, 3 ml of peripheral whole blood was aspirated from the left cubital fossa veins, directly into an EDTA containing tube. These blood samples were subjected to Trizol preservation.

## Total RNA extraction with TRIzol

Total RNA of all samples was extracted using the TRIzol® LS Reagent following the protocol provided by the manufacturer (10).

## cDNA synthesis

Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl). Thermal cycler steps of conditions cDNA Reverse Transcription are show in (Table 1). Primers and Probes used in the study are shown in (Table 2) for *GAPDH* and *LUNX* (11, 12).

**Table (1): Conditions of Primers Thermal cycler steps for cDNA Reverse Transcription.**

| | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| Temperature (C°) | 25 | 42°C | 85 | 4 |
| Time (min) | 10 | 30 | 5 | ∞ |

**Table (2): Primers used for *GAPDH* and *LUNX***

| Primer | Sequence (5′→3′ direction) |
|---|---|
| ***LUNX*** | |
| Forward | AATGAGGTTCTCAGAGGCTT |
| Reverse | TTAGACCTTGATGACAAACT |
| ***GAPDH*** | |
| Forward | GAAATCCCATCACCATCTTCCAGG |
| Reverse | GAGCCCCAGCCTTCTCCATG |

**Reverse transcription polymerase chain reaction (RT–pcr)**

RT-PCR was performed using the stratagene Real-time PCR System (Analytik Jena Technologies) with qPCRsoft software. The gene expression levels and fold change were quantified by measuring the cycle threshold (Ct) employing the 2xqPCR Master Mix Kits components. Every reaction was done in a duplicate and included a non-template control (NTC), non-amplification control (NAC) and non-primer control (NPC) as negative controls.

**Table (3): Component of real-time PCR used in *GAPDH* and *LUNX* genes expression experiment.**

| Reagent Master Mix | Final conc. | 20 µl rxn |
|---|---|---|
| Forward Primer (10 µM) | 300nM | 0.6 µl |
| 2xqPCR Master Mix | 1x | 10 µl |
| PCR grade water up to 20 µl | | 6.8 |
| Reverse Primer (10 µM) | 300nM | 0.6 µl |
| Template DNA | (<20 ng/20 µl rxn) | 2 µl |

## The qPCR Reaction run

The cycling protocol was programmed according to the thermal profile Shown in the (Table 4), (Figure 1) for *LUNX* gene.

**Table (4): Thermal profile of *LUNX* genes expression**

| Step | Temp. | Duration | Cycles |
|---|---|---|---|
| **Enzyme activation** | **95°C** | **10 min** | **Hold** |
| **Denature** | **95°C** | **5sec** | **40** |
| **Anneal/ extend** | **58°C** | **30 sec** | |
| **Dissociation** | **1min /95 °C-30 sec /55 °C-30sec/95 °C** | | |

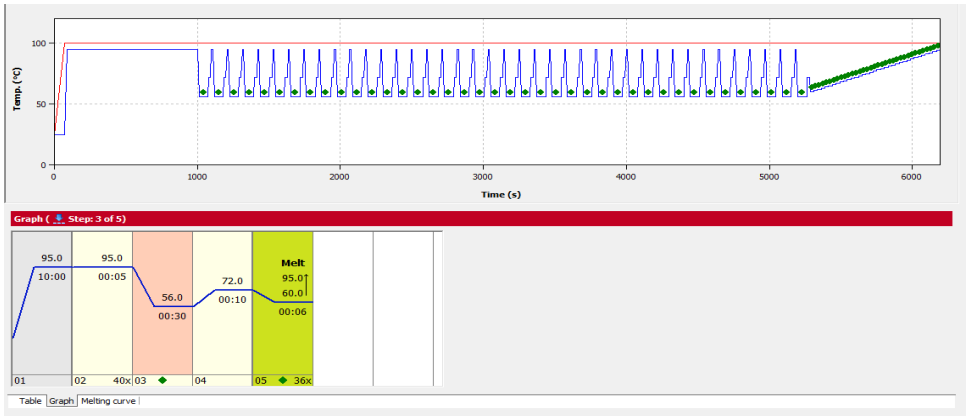


**Figure (1): Thermal profile used in expression of LUNX genes. The profile was taken directly from RT-PCR machine.**

## Housekeeping Gene Amplification

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control to be used in calculating the ΔCT value. A qPCR reaction of amplification of *GAPDH* was done with the Thermal profile shown in (Table 5), (Figure 2) .

**Table (5): Thermal profile of *GAPDH* gene expression**

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Enzyme activation | 95ºC | 3 min | Hold |
| Denature | 95ºC | 5 sec | 40 |
| Anneal/extend | 60ºC | 20 sec | |
| Dissociation | 1min /95 ºC-30 sec /55 ºC-30sec/95 ºC | | |

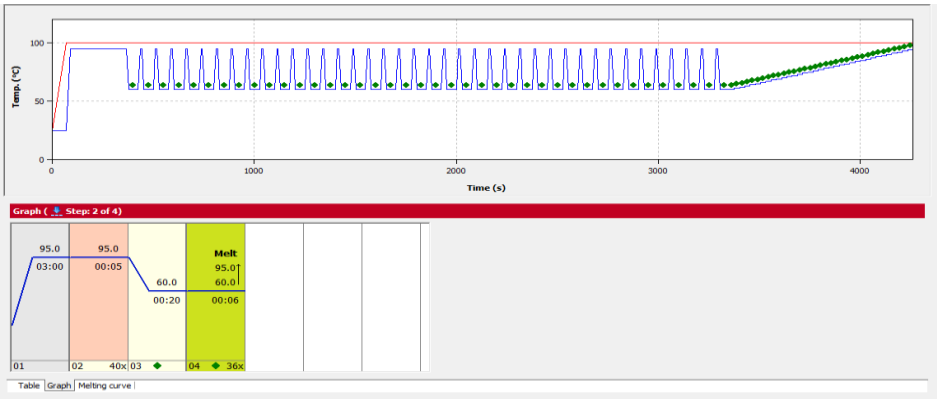


**Figure (2): Thermal profile used in *GAPDH* Amplification .The photograph was taken directly from qPCR machine.**

## Real Time RT-PCR analysis of *LUNX* and *GAPDH* genes expression.

### 1. Using ΔCT

The expression ratio was calculated without a calibrator sample 2-ΔCt according to the following equation:
ΔCT (test) = CT of gene of interest (target, test) – CT internal control
Finally, the expression ratio was calculated according to the formula $2^{-\Delta Ct}$ = Normalized expression ratio.

### 2. Using ΔΔ CT

To compare the transcript levels between different samples the $2^{-\Delta\Delta Ct}$ method was used [13].
The CT of gene of interest was normalized to that of internal control gene. The difference in the Ct values between the *GAPDH* (internal control gene) and a *LUNX* gene (interest gene) was calculated as the following formula:
ΔCT (test) = CT gene of interest (target, test) – CT internal control
ΔCT (calibrator) = CT gene of interest (target, calibrator) – CT internal control.
The calibrator was chosen from control samples.
CT values ≥ 38 were considered unreliable and neglected
The ΔCT of the test samples was normalized to the ΔCT of the calibrator:
ΔΔ CT was calculated according to the following equation:
ΔΔ CT=ΔCT (test) - ΔCT (calibrator)
Finally, the expression ratio was calculated according to the formula
$2^{-\Delta\Delta Ct}$ = Normalized expression ratio.

## Result and Discussion

The Ct value of *GAPDH*, the housekeeping gene used in the present

study is shown in (Table 6). The range of Ct value for *GAPDH* in Lung cancer smoker patients group was 24.10-25.10 with a mean±SD **(23.33±0.28)**, for the lung cancer non-smoker patients group it ranged from 24.27-25.01 with a mean±SD **(23.42±0.31)**. On the other hand, among the healthy smoker group the range was from 24.18-25.04 with a mean±SD **(23.53±0.27)** versus 24.30-24.88 with a mean±SD **(23.42±0.29)** in the Healthy nonsmoker group. Non-significant difference was found in between these groups regarding the mean Ct value of *GAPDH*, (p=0.49: p<0.05) with an LSD value of (1.59).

**Table (6): Comparison between the different studied groups in the *GAPDH* Ct value of (Mean±SD)**

| Group | No. | Mean ± SD of Ct value | Range |
|---|---|---|---|
| **Lung cancer smoker** | 30 | 23.42±0.29 | 22.30-24.04 |
| **Lung cancer non-smoker** | 30 | 23.53±0.27 | 22.29-24.07 |
| **Healthy smoker** | 40 | 23.42± 0.31 | 22.33-24.00 |
| **Healthy non-smoker** | 40 | 23.33±0.28 | 22.30-24.10 |
| **LSD** | | 1.59 | |
| **P-value** | | 0.49 | |

The inherent assumption in the use of housekeeping genes in molecular studies is that their expression remains constant in the cells or tissue under investigation (14).

This result was not surprising, as *GAPDH* is one of the most commonly used housekeeping genes in companion of gene expression data is *GAPDH* (15).

Robert *et al*. (16) studied the expression of 1,718 genes using RT-PCR by appling the *GAPDH* as a reference gene in 72 kinds of normal human tissue. They found that using of *GAPDH* is quite a reliable strategy for the normalization in qRT-PCR when applied in clinical studies.

To further improve this and although there was a significant difference in the mean Ct value between groups in the present study, the variation of total change in expression of *GAPDH* was studied in different study groups utilizing the $2^{-Ct}$ value and the ratio of $2^{-Ct}$ of the different study groups to that of control group, as shown in (Table 7).

**Table (7): Comparison of *GAPDH* fold expression between the study groups.**

| Group | Means Ct of *GAPDH* | $2^{-Ct}$ | experimental group/ Control group | Fold of gene expression |
|---|---|---|---|---|
| **Lung cancer smoker** | 24.34 | 4.70 E-8 | 4.70 E-8/4.45 E-8 | 1.05 |
| **Lung cancer non-smoker** | 24.42 | 4.45 E-8 | 4.45 E-8/4.45 E-8 | 1.00 |
| **Healthy smoker** | 24.53 | 4.12 E-8 | 4.12 E-8/4.45 E-8 | 0.93 |
| **Healthy non-smoker** | 24.42 | 4.45 E-8 | 4.45 E-8/4.45 E-8 | 1.00 |

The $2^{-Ct}$ value in Lung cancer smoker was 4.70 E-8, for Lung cancer nonsmoker it was 4.45 E-8, while in healthy smoker it was equivalent to 4.12E-8 and in healthy nonsmoker it was 4.45 E-8. The computed ratio for gene fold expression was 1.05, 1.00, 0.93 and 1.00 respectively. These small variations in gene fold expression between the study groups renders *GAPDH* gene a useful control gene.

The mean±SD Ct value of *LUNX* cDNA amplification was (27.25± 0.42) in the patients Lung cancer smoker. The Ct values in patients Lung cancer nonsmoker was a mean±SD (27.47±0.32). While Ct values in Healthy smoker, healthy nonsmoker were mean±SD (29.33±0.39) and mean±SD (30.71±0.45) respectively. Results are shown in (Table 8). There was a significant difference in the mean Ct values between the different study groups (p=0.0001).

Expression of the *LUNX* gene was equal in both smoker Lung cancer patients and Lung cancer nonsmoker and this in turn was higher than those in healthy smoker, the latter being higher than healthy nonsmoker. This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the patients group is associated with the highest copy number of mRNAs reflecting its higher expression. Lung cancer explains this difference in Ct values between the two groups in this study.

On the other hand, exposure to smoking causes different Ct values for the normal groups.

The results show convergence of Ct values between the lung cancer smoker and lung cancer non-smoker groups and it is important evidence that *LUNX* gene expression increases in both patients groups so it is possible to use *LUNX* gene as a biomarker for the early detection of lung cancer.

These results agreed with those registered by Shirin *et al*. (17). Their results indicate that the mentioned mRNA could be suggested as sensitive and specific markers in peripheral blood for the primary diagnosis of lung cancer. The findings are consistent as well with those of Zheng *et al*. (18) whose results demonstrated that the *LUNX* as a molecule overexpressed in primary NSCLC and lymph node metastases that is associated with reduced postoperative survival. These results coincide with those reported by Xiansen *et al.* (19) which revealed that the mRNA expression of *LUNX* in the peripheral blood is of a significant clinical value for the diagnosis of lung cancer. Within that respect, Cheng *et al*., (20) who provided a detailed evaluation of the lung cancer tumor markers of *LUNX* mRNA and assessed the diagnostic utility of this marker in patients with NSCLC. The results indicated that *LUNX* mRNA was the most specific gene marker for lung cancer and had potential diagnostic utility when measured in the peripheral blood with NSCLC. Yang *et al.,* (21) revealed that *LUNX* mRNA was detected in the peripheral blood and regional lymph nodes, and that there was no expression of *LUNX* mRNA in benign lung diseases and the peripheral blood and lymph nodes of healthy people. Therefore, the evaluation of mRNA expression of *LUNX* in the peripheral blood had important clinical value for the diagnosis and the prognosis of lung cancer.

**Table (8): Comparison between Ct, ΔCt and 2-ΔCt (Mean ± SD) value of *LUNX* gene in different groups**

| Group | No | Mean ± SD of Ct | Mean ± SD ΔCt | Mean ± SD 2-ΔCt |
|---|---|---|---|---|
| Lung cancer smoker | 40 | 27.25±0.42 | 2.91±1.06 | 0.133±0.33 |
| Lung cancer non-smoker | 40 | 27.47±0.32 | 3.05±1.61 | 0.120±0.40 |
| Healthy smoker | 30 | 29.33±0.39 | 4.80±2.18 | 0.036± 0.31 |
| Healthy non-smoker | 30 | 30.71± 0.45 | 6.29 ± 2.43 | 0.012 ± 0.02 |
| LSD value | --- | 0.551 | 2.55 | 0.43 |
| P-value | --- | 0.006 | 0.021 | 0.001 |

Each quantificative PCR reactions was run in duplicate for each sample**.** In each run, samples from Lung cancer smoker, Lung cancer nonsmoker, Healthy smoker and Healthy smoker were run in addition to non-template and non- primer controls. This was important to make the statistical calculation of each group and in order to specify the calibrator.

In the present study, RT-PCR assay analyzed the mRNA expression of ***LUNX*** and compared its expression between apparently healthy nonsmoker group, Healthy smoker group, Lung cancer smoker group and Lung cancer non-smoker group. The calculation of gene expression fold change was made using relative quantification (22).

This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of ***LUNX*** cDNA amplification of each single case and that of the ***GAPDH***.

(Table 9) shows the mean of ΔCt (normalization Ct values) of each study group. ΔCt means in Groups Lung cancer smoker, Lung cancer non-smoker, healthy smoker and healthy non-smoker were **(2.91)**,

**(3.05), (4.80)** and **(6.29)**, respectively. A significant difference was noticed between the study groups ($p=0.001$; $p<0.05$).

Results of $\mathbf{2^{-Ct}}$ revealed significantly ($p=0.0001$; $p<0.05$), higher results for the Lung cancer smoker group **(0.133**) from the other three groups including Lung cancer non-smoker group, healthy smoker group and healthy non-smoker group **(0.12) (0.036) (0.012),** respectively.

To calculate the gene expression folds in relation to the housekeeping genes the result of $\mathbf{2^{-Ct}}$ of each group was measured in relation to that of Healthy nonsmoker group. Results are shown in (Table 9). The fold of gene expression in groups, healthy smoker, healthy non-smoker, Lung cancer non-smoker and Lung cancer smoker were **(1.0)**, **(3.0), (10.0)** and **(11.0)**, respectively. These results indicate significantly increase expression of *LUN. X* gene in these groups.

**Table (9): Fold of *LUNX* expression Depending on $2^{-Ct}$ Method**

| Groups | Means Ct of *LUNX* | Means Ct of *GAPDH* | ΔCt (Means Ct of *LUNX* - Means Ct of *GAPDH*) | $2^{-Ct}$ | experimental group/ Control group | Fold of gene expression |
|---|---|---|---|---|---|---|
| Lung cancer smoker Group 1 | 27.25 | 24.34 | 2.91 | 0.133 | 0.133/0.012 | 11.00 |
| Lung cancer non-smoker Group 2 | 27.47 | 24.42 | 3.05 | 0.120 | 0.120/0.012 | 10.00 |
| Healthy smoker Group 3 | 29.33 | 24.53 | 4.80 | 0.036 | 0.036/0.012 | 3.00 |
| Healthy non-smoker Group 4 | 30.71 | 24.42 | 6.29 | 0.012 | 0.012/0.012 | 1.00 |

In calculation of the relative expression of ***LUNX*** gene in all study groups the 2-ΔΔCt results was applied. A calibrator was used and it was one of the samples of the controls with high expression of ***LUNX***.

As shown in (Table 10), there was a significant difference (p=0.0001). among the four groups Lung cancer smoker group, Lung cancer nonsmoker group, healthy nonsmoker group and healthy smoker group with regarding to the mean of $2^{-\Delta\Delta Ct}$ (36.00), (32.6), (9.71) and (3.45), respectively.

**Table (10): Fold of *LUNX* expression Depending on 2-ΔΔCt Method**

| Groups | Means Ct of *LUNX* | Means Ct of *GAPDH* | Mean ΔCt Target (ct *LUNX* -ct *GAPDH)* | Mean ΔCt Calibrator (ct *LUNX* -ct *GAPDH* | ΔΔCt | 2-ΔΔCt | experimental group/ Control group | Fold of gene expression |
|---|---|---|---|---|---|---|---|---|
| Lung cancer smoker | 27.25 | 24.34 | 2.91 | 8.08 | -5.17 | 36.00 | 36.00/3.24 | 10.5 |
| Lung cancer non-smoker | 27.47 | 24.42 | 3.05 | 8.08 | -5.03 | 32.6 | 38.85/3.45 | 9.5 |
| Healthy smoker | 29.33 | 24.53 | 4.80 | 8.08 | -3.28 | 9.71 | 9.71/3.45 | 2.9 |
| Healthy non-smoker | 30.71 | 24.42 | 6.29 | 8.08 | -1.79 | 3.45 | 3.45/3.45 | 1.00 |

When calculating, the gene expression it was significantly higher in Lung cancer smoker than Healthy nonsmoker group, 10.5 times. Fold number in Lung cancer nonsmoker group was 9.5 times than the Healthy nonsmoker, Fold number in Healthy smoker group was 2.9 times than the Healthy nonsmoker group, as shown in (Table 10).

Statistical results show there is a high correlation between the *LUNX* gene expression and lung cancer, where the correlation coefficient was 0.879 and the show a low correlation between *LUNX* gene expression and smoking, where the correlation coefficient was 0.446.

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