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Expression of *APEX1* Gene in Specimens of Iraqi Patients with Lung Cancer

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Abstract : The etiology of lung cancer has been shown to be associated with genetic and certain environmental factors that produce DNA damage. Base excision repair (BER) genes are responsible for repair of DNA damage caused by reactive oxygen species and other electrophiles and thus have been reported to be good candidate susceptibility genes for lung cancer. Apurinic/apyrimidinic endonuclease-1 (*APEX1*) proteins have important functions in the BER pathway. Increased levels of *APEX1* in cancer have been reported. However, available methods for measuring *APEX1* levels are direct and quantitative by using real time Poly Chain Reaction (RT-PCR).

In the present study, whole blood was isolated from 140 individuals distributed into four groups as follows:Group 1 included: 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 non-smokers apparently healthy individuals. The messenger RNA (mRNA) expression levels of *APEX1*in the peripheral blood were analyzed using reverse transcription‑polymerase chain reaction (RT‑PCR). The expression of *APEX1* mRNA in the Groups 1, 2, 3 and 4 were 16.57, 12.0, 4.0and 1.0 folds of the gene expression, respectively, Using GAPDH as Housekeeping Gene.

In conclusion, the existence of a significant correlation between blood and tumor tissue expression of *APEX1*gene in lung cancer, could allow the introduction in clinical practice of a simple test that would measure mRNA levels of DNA repair genes in peripheral blood samples instead of tissue samples; thus justifying its use as a prognostic and predictive factors in lung cancer patients.

Key Words : mRNA, *APEX1* Gene, RT-PCR, Lung Cancer, *GAPDH.*

Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women in many countries throughout the world[1]. The majority of cases (~60%) are diagnosed after the disease has spread to distant organs 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[2].

The majority of cases of lung cancer are due to long-term tobaccosmoking, 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 [3][4].

Apurinic/apyrimidinic endonuclease 1 (*APEX1*) is an essential enzyme in the base excision repair pathway, which is the primary mechanism for the repair of DNA damage. Diseases associated with *APEX1* include attenuated familial adenomatous polyposis and respiratory failure. Among its related pathways are Cell Cycle / Checkpoint Control and Spinal Cord Injury [5].

Apurinic/apyrimidinic (AP) sites occur frequently in DNA molecules by spontaneous hydrolysis, by DNA damaging agents or by DNA glycosylases that remove specific abnormal bases. AP sites are pre-mutagenic lesions that can prevent normal DNA replication so the cell contains systems to identify and repair such sites. Class II AP endonucleases cleave the phosphodiester backbone 5' to the AP site. This gene encodes the major AP endonuclease in human cells. Splice variants have been found for this gene; all encode the same protein[6].

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) 40 Samples from smoker patients affected by lung cancer.
2) 40 Samples from non-smoker patients affected by lung cancer.

Group 2: Sixty apparently healthy individuals of both sexes at different ages. They were subdivided into two groups:-

3) 30 Samples of smokers apparently healthy individuals.
4) 30 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 presevation.

Total RNA extraction with TRIzol

Total RNA of all samples was extracted using the TRIzol® LS Reagent following the protocol provided by the manufacturer [7].

cDNA synthesis for mRNA

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).

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

Step 4	Step 3	Step 2	Step 1	
4	**85**	**42**	**25**	**Temperature(ºC)**
∞	**5**	**30**	**10**	**Time(min)**

Primers and Probes used in the study are shown in (Table 2) for *GAPDH* and *APEX1*[8] [9].

Table (2): Primersused in the study

Primer	Sequence (5′→3′ direction)
APEX1	
Forward	**AAACGAGTCAAATTCAGCCACAA**
Reverse	**CCCACCTCTTGATTGCTTTCC**
GAPDH	
Forward	**GAAATCCCATCACCATCTTCCAGG**
Reverse	**GAGCCCCAGCCTTCTCCATG**

Real Time PCR (qRT–PCR)

QRT-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 threshold cycle (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 quantitative real-time PCR used in *GAPDH* and *APEX1* genes expression experiment.

Reagent Master Mix	Final concentration	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 *APEX1* gene.

Table (4): Thermal profile of *APEX1* genes expression

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	10 min	Hold
Denature	95ºC	10 sec	40
Anneal/extend	62ºC	30 sec	
Dissociation	1min /95 ºC-30 sec /55 ºC-30sec/95 ºC		

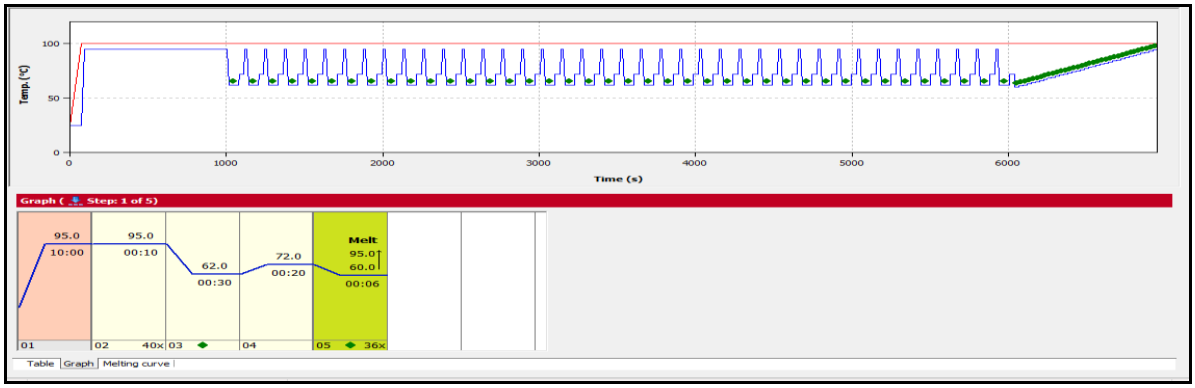

Figure (1): Thermal profile used in expression of *APEX1* genes. The profile was taken directly from qPCR machine

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		

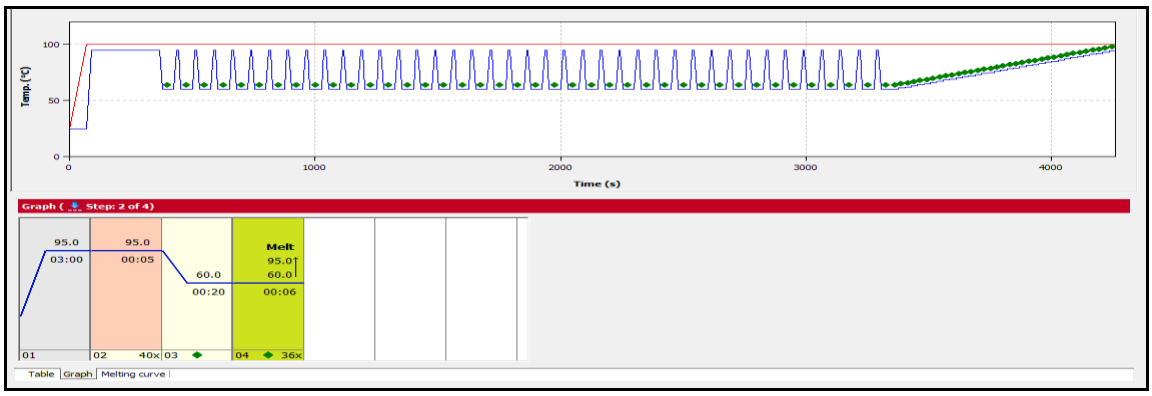

Figure (2): Thermal profile used in *GAPDH* Amplification.The photograph was taken directly from qPCR machine.

Real Time qRT-PCR analysis of *APEX1* and *GAPDH* genes expression

1. ΔCT

The expression ratio was calculated without a calibrator sample 2-ΔCt according to the following equation:

ΔCT (test) = CT 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. ΔΔ CT

To comparethe transcript levels between different samples the $2^{-\Delta\Delta Ct}$ method was used [10].

The CT of gene of interest was normalized to that of internal control gene. The differencein the cycle threshold (Ct) values between the *GAPDH*(internal control gene) anda *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 group 1 was 24.10-25.10 with a mean±SD **(23.33±0.28)**, for group 2,group 3 and group 4 it ranged from 24.27-25.01 with a mean±SD **(23.42±0.31)**,24.18-25.04 with a mean±SD **(23.53±0.27)** and24.30-24.88 with a mean±SD **(23.42±0.29), resp.**Non-significant difference was found in between these groups regarding the mean Ct value of *GAPDH*, (p=0.046: p<0.05) with an LSD value of (0.256).

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

Groups	No.	Mean ± SD of Ct value	Range
group 4	30	23.42±0.29	22.30-24.04
group 3	30	23.53±0.27	22.29-24.07
group 2	40	23.42± 0.31	22.33-24.00
group 1	40	23.33±0.28	22.30-24.10
LSD		0.256	
P-value		0.046	

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 [11].

This result was not surprising, as *GAPDH* is one of the most commonly used housekeeping genes in companion of gene expression data is *GAPDH*[12].

Robert *et al.* [13] 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 thedifferent study groups to that of control group, as shown in (Table 7).

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

Groups	Means Ct of *GAPDH*	2^{-Ct}	experimental group/ Control group	Fold of gene expression
Group 1	24.34	4.70 E-8	4.70 E-8/4.45 E-8	1.05
Group 2	24.42	4.45 E-8	4.45 E-8/4.45 E-8	1.00
Group 3	24.53	4.12 E-8	4.12 E-8/4.45 E-8	0.93
Group 4	24.42	4.45 E-8	4.45 E-8/4.45 E-8	1.00

The 2^{-Ct} value in group 1 was 4.70 E-8, for group 2 it was 4.45 E-8, while in group 3 it was equivalent to 4.12E-8 and in group 4 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 ***APEX1*** cDNA amplification was **(27.44± 0.98)** in the group 1. The Ct value in group 2 was a mean ± SD **(27.99±1.35).** While the Ct values in group 3 and group 4 were mean±SD **(29.68±1.19) and** mean ± SD **(31.88±1.22),**respectively. The results are shown in (Table 8). There was a significant difference in the mean Ct values between the different study groups (p=0.0001).

The mean Ct values of group 1 were higher than those with group 2, this in turn was higher than group 3 which was higher than group 4. 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.As a result of smoking these differences appeared in CT values. The results show differences in CT values which were high for Lung cancer smoker group.

This result showed that the gene had a high expression due to the damage caused by smoking on the genetic content.

These results agreed with those reported by Xiaoliang *et al.* [8] which observed that thepolycyclic aromatic hydrocarbons (PAHs) are the most significant contributors to tobacco-induced lung carcinogenesis**.***APEX1*is a central enzyme in the removal of apurinic/apyrimidinic sites caused by DNA damaging agents. This study aimed to investigate the potential interaction of *APEX1* polymorphisms and PAHs on genetic damage and lung cancer risk,

Our findings are consistent as well with those displayed by Marina *et al.*[14]Their results indicate that the existence of a significant correlation between blood and tumor tissue expression of some genes of clinical interest, such as *APEX1*in NSCLC and HNSCC, could allow the introduction in clinical practice of a simple test

that would measure mRNA levels of DNA repair genes in peripheral blood samples instead of tissue samples to determine prognostic and predictive factors in NSCLC and HNSCC patients.

Table (8): Comparison between different groups in Ct, ΔCt and $2^{-\Delta Ct}$ value (*APEX1*) (Mean ± SD)

Groups	No	Mean ± SD of Ct	Mean ± SD ΔCt	Mean ± SD 2^{-Ct}
Group 1	40	27.44±0.98	3.1±1.02	0.116±0.011
Group 2	40	27.99±1.35	3.57±1.33	0.084±0.016
Group 3	30	29.68±1.19	5.15±0.89	0.028±0.017
Group 4	30	31.88±1.22	7.1±0.97	0.014±0.009
LSD value	---	1.66	1.51	0.197
P-value	---	0.001	0.001	0.001

Each quantificativePCR reactions was run in diplicate for each sample, In each run , samples fromgroup 1, group 2, group 3 and group 4 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. Plots of each run were recorded including the amplification plots and dissociation curves.

In the present study, quantitative RT-PCR assay analyzed the mRNA expression of ***APEX1***and compared its expression between apparently group 4, group 3, group 2 and group 1. The calculation of gene expression fold change was made using relative quantification [10].

This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *APEX1*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 group 1, group 2, group 3 and group 4 were **(3.1)**, **(3.57), (5.15)** and **(7.1)** respectively. A significant difference was noticed between the study groups (p=0.001).

Results of 2^{-Ct} revealed significantly higher results for the group 1 from the other three groups (p=0.0001), mean of 2^{-Ct} for group 1**(0.116**). In the group 2, group 3 and group 4 a mean of 2^{-Ct} were **(0.084)(0.028)(0.014),** respectively.

To calculate the gene expression folds in relation to the housekeeping genes the result of 2^{-Ct} of each group was measured in relation to that of Healthy nonsmoker group. The results are shown in (Table 9).The fold of gene expression in group 1 was higher than group 4 in **16.57** times. Fold number in group 2 was **12.0** times as the group 4,fold number in group 3 was **4.0** times as the group 4 as shown in (Table 9). These results indicate a significantly increase expression of *APEX1*gene in these groups.

Table (9): Fold of *APEX1* expression Depending on $2^{-\Delta Ct}$Method

Groups	Means Ct of *APEX1*	Means Ct of *GAPDH*	ΔCt (Means Ct of *APEX1* - Means Ct of *GAPDH*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 1	27.44	24.34	3.1	0.116	0.116/0.007	16.57
Group 2	27.99	24.42	3.57	0.084	0.084/0.007	12.00
Group 3	29.68	24.53	5.15	0.028	0.028/0.007	4.00
Group 4	31.88	24.42	7.1	0.014	0.007/0.007	1.00

The calculation of the relative expression of *APEX1*gene in all study groups was done by applying the $2^{-\Delta\Delta Ct}$ results. A calibrator was used which was one of the samples of the controls with high expression of *APEX1*.

As shown in (Table 10), the mean of $2^{-\Delta\Delta Ct}$ values of group 1, group 2, group 3 and group 1 it was (51.26), (37.01), (12.38) and (3.20), respectively. There was a significant difference between these groups regarding the mean $2^{-\Delta\Delta Ct}$, (p=0.0001).

Table (10): Fold of *APEX1* expression Depending on 2-ΔΔCt Method

groups	Means Ct of *Apex1*	Means Ct of *GAPDH*	Mean ΔCt Target (ct *Apex1* -ct *GAPDH)*	Mean ΔCt Calibrator (ct *Apex1*-ct *GAPDH*	ΔΔCt	2-ΔΔCt	experimental group/ Control group	Fold of gene expression
Group 1	27.44	24.34	3.1	8.78	-5.68	51.26	51.26/3.20	16.01
Group 2	27.99	24.42	3.57	8.78	-5.21	37.01	37.01/3.20	11.6
Group 3	29.68	24.53	5.15	8.78	-3.63	12.38	12.38/3.20	3.9
Group 4	31.88	24.42	7.1	8.78	-1.68	3.20	3.20/ 3.20	1.00

When calculating gene expression it was significantly higher in group4than group1**16.01** times. Fold number in group 2 was **11.6** times then the group 4, Fold number in group 3 was **3.9** times then the group 1 as shown in (Table 10). The above results demonstrate the significant gene expression in group 1.

All study groups were divided into two subgroups, high expression when the fold change of gene expression was above 1 and low expression when the fold change was lower than 1.

The frequency of high expressinggroup 1was 47.5%, for group 2 it was 40.0%, for group 3 it was 23.3%, and for group 1at control group it was 0.0% for gene*APEX1*respectively, as shown in (Table 11).

Table (11): Frequency of high and low expression in study groups for *APEX1*gene.

Low <1	High >1 n (%)	
21(52.5%)	19(47.5%)	Group 1
24(60.0%)	16(40.0%)	Group 2
23(67.7%)	7(23.3%)	Group 3
30 (100%)	0.0(0%)	Group 4
Group 1and group 4		
19.5588		Chi square
0.00001		P value
Group 2 and group 4		
16.355		Chi square
0.000053		P value
		Group 3 and group 4
7.9245		Chi square
0.0048		P value

It is well shown here that the high expression was evident in group 1 in comparison to the group 4. However, group 2 also show a good number of high expressing individuals. Induction of *APEX1* gene expression is apparently partly due to the cancer itself. There was a significant statistical difference between the study groups, $p<0.05$ suggested the importance of detecting the high expression of *APEX1* gene as a marker for DNA damage and repair, as an important conclusion of this experiment to be conducted on all lung cancer

smoker and non-smoker. This gene expression study must be conducted annually as an important follow up investigation.

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