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Design Of Primer And Probe To Detect SNP Rs 1892901 In Fosl-1Gene In Different Types Of Cancer In Iraqi Population

Safa Ahmed Abed *1 Hiba Muneer Al-Khafaji2

1,2 Department of Applied Sciences / University of Technology/Iraq
Corresponding Author: safaahmedabd96@gmail.com

ABSTRACT

It has been shown that the etiology of thyroid, breast and prostate cancer is associated with hereditary and some environmental factors that cause damage to DNA. Protein coding genes are responsible for the development of protein and thus have been reported to be good candidate susceptibility genes for thyroid, breast and prostate cancer. (FOSL1) proteins have important functions in the growth, including the regulation of cell proliferation, differentiation and transformation. There have been studies of elevated levels of FOSL1 in cancers. However, the available methods for measuring FOSL1 levels are direct and quantitative using Poly Chain Reaction (RT-PCR) in real time. In the present study, whole blood was isolated from 100 individuals distributed into four groups as follows: Group 1 included: 25 samples from thyroid cancer patients; Group 2: 25 samples from breast cancer patients; Group 3: 25 samples from prostate cancer and Group 4:25 samples from apparently healthy individuals. The messenger RNA (mRNA) expression levels of FOSL_1in the peripheral blood was analyzed using reverse transcription polymerase chain reaction (RT PCR). The expression of FOSL_1 mRNA in the fold of gene expression in prostatic cancer group was 4 time higher than that of healthy group. That for the breast cancer group was 3 times higher than the healthy group and the thyroid cancer group was 3 times higher than the healthy group., respectively, Using GAPDH as Housekeeping Gene. In conclusion, there is an important link between blood and tumor tissue expression of FOSL1 gene. in thyroid, breast and prostate cancer, could allow the introduction in clinical practice of a simple test that would measure mRNA levels of DNA protein coding genes in peripheral blood samples instead of tissue samples; thus justifying its use as a prognostic and predictive factors in thyroid, breast and prostate cancer patients.

Keywords: Design Of Primer And Probe To Detect SNP, Fosl-1Gene In Different Types Of Cancer, Iraqi Population

Correspondence:
Safa Ahmed Abed
Department of Applied Sciences / University of Technology/Iraq

*Corresponding author: Safa Ahmed Abed email-address: safaahmedabd96@gmail.com

INTRODUCTION

Cancer is one of the important health problems of the current era and also a leading cause of death among populations. [1][2] the thyroid cancer, which is known to be the most common type of endocrine malignancies, which also represents less than 1% of all human tumors. The incidence of thyroid cancer varies greatly depending on gender, age and geographic region. [3][4] Despite of rise in its rates of thyroid cancer, but the mortality has not risen in equal measure. However, the mortality are higher two times in female. [5][6] Another type of cancer is prostate cancer, which is known to be the most common male cancer, and is the main cause of death in men diagnosed with this type of cancer. [7] the breast cancer (bc), is consider the second most common cancer worldwide and the most frequently diagnosed life-threatening cancer in women. [8] Approximately 5–10% of cases are the result of a genetic predisposition inherited from a person's parents. [9] FOS-like antigen 1 (FOSL1) recognized as the transcription factor for leucine zipper. The FOSL1 gene is protein coding gene plays a key role in cellular signs of growth, including the regulation of cell proliferation, differentiation and transformation. [12]Many studies focused on the role of FOSL1 gene in association with cancer. However, best to the present knowledge, this is the first study in Iraq to tackle the association of fosl_1 gene polymorphism and its mRNA expression.

MATERIALS AND METHODS

This study was taken place during the period from October 2019 to May 2020. All the study experiments were performed at the University of Technology and Iraqi Hereditary company (IHC).

STUDY GROUPS

The total number of participants in the study was 100 individuals**,** study groups included the following: -

GROUP 1: Seventy-five patient's samples of Iraqi man and women diagnosed with different types of cancer, aged between (21-78 years) the samples were collected at Al-Amel National Hospital for Cancer Treatment in Baghdad/Iraq, their clinical information was obtained from their hospital files and case-sheet records; divided into: -

a- 25 Samples from patients with thyroid cancer.
b- 25 samples from patients with breast cancer.
c- 25 samples from patients with prostate cancer.

GROUP 2: Twenty-five samples of apparently healthy individuals of both sexes, aged between (19-70 years) were obtained for control.

BLOOD SAMPLING

From each participant, 3 ml of whole blood was needed to be collected from the venous blood directly into an EDTA containing tube**,** this procedure was done under aseptic conditions.

TOTAL RNA EXTRACTION WITHOUT TRIZOL

The RNA extraction from whole blood of both patient and healthy control without TRIzol subjects by using protocol in *EasyPure*® Blood RNA Kit.

CDNA SYNTHESIS FOR MRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using *EasyScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix 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.

	Step1	Step2	Step3
Temperature	25°c	42°c	85°c
Time	10min	15min	5seconds
	Random Primer (N9)	Anchored Oligo(dT)18	Inactivate reverse transcriptase enzyme

Primers and Probes used in the study are shown in (Table 2) for GAPDH and FOSL_1.

Table (2): primers and probes

Primer/probe	Sequence (5′→3′ direction)
FosL1 gene expr.	
Forward	CACCCTAGCCAATGTCTCCT
Reverse	AGAAACAGTGGGCAGCTTTG
GAPDH house-keeping gene	
Forward	TGAGAAGTATGACAACAGCC
Reverse	TCCTTCCACGATACCAAAG

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 FOSL_1 genes expression experiment.

Component	**Volume**
Master mix EasyTaq® PCR SuperMix	12.5µl
Forward primer	1 µl
revers primer	1 µl
DNA	4µl
Nuclease free water (N.F.W)	6.5µl

THE QPCR REACTION RUN

The cycling protocol was programmed according to the thermal profile Shown in the (Table 4).

Table (4): Thermal profile of FOSL_1 genes expression

		Temperature	Time	cycle
Stage 1	Denaturation	95ºC	5 m	1
Stage 2	Denaturation	95ºC	30 S	40
	annealing	58ºC	40 S	
	Extension	72ºC	40 S	
Stage 2	Extension	72ºC	10 m	1

HOUSEKEEPING GENE AMPLIFICATION

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

Table (5): Thermal profile of GAPDH gene expression

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

REAL TIME QRT-PCR ANALYSIS OF FOSL_1 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 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 cycle threshold (Ct) values between the housekeeping gene (internal control gene) and FOSL1 genes (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 the 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 the healthy group was **23.09-23.2**with a mean ± SD **(23.125±0.05)**. For the prostate cancer group, it ranged from **23.05-23.23** with a mean ± SD **(24.6±0.37)**. In the breast cancer group, it ranged from **23.01-23.14** with a mean ± SD **(23.11±0.05).** In thyroid group it ranged from **23.04-23.22** with a mean ± SD **(23.1±0.07)**. A significant difference was found in between these groups regarding the mean Ct value of *GAPDH*, (p= **0.732**: p<0.05) with an LSD value of (**2.644**).

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

Group	No.	Mean ± SD of Ct value	Range
Group 1 Healthy	25	23.125±0.05	23.09-23.2
Group 2 Prostatic Cancer	25	23.09±0.06	23.05-23.23
Group 3 Breast Cancer	25	23.11±0.05	23.01-23.14
Group 4 Thyroid Cancer	25	23.1±0.07	23.04-23.22
LSD		2.644NS	
P-value		0.732	

NS: Non-significant.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. One of the most commonly used housekeeping genes in companion of gene expression data is *GAPDH*.[14] studied the expression of 1,718 genes using qRT-PCR they applied 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 Table7. The 2^{-Ct} value of healthy group was **1.093 E-7**. For prostate cancer group, it was **1.119 E-7** and for breast cancer group it was **1.104 E-7**.and for thyroid group it was **1.112 E-7.** The computed ratio for gene fold expression was **1.000** for the healthy groups, **1.023** for the prostatic cancer group and **1.010** for the breast cancer group. **1.017** for the thyroid cancer group. These small variations in gene fold expression between the study groups renders *GAPDH* gene a useful control gene.The Ct value of FOSL1 **cDNA** amplification was **ranged from 21.97 to 23.75** in healthy group. It ranged from **20.48 to 21.01** in thyroid cancer group. The range of Ct values in breast cancer group was from **20.22 to 20.63**. While Ct values ranged from **19.42 to 20.62** in the prostatic cancer group.The mean Ct values of prostate cancer group were higher than those with breast cancer group, this in turn was higher than thyroid cancer group which was higher than the healthy group. 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 cancer these differences appeared in CT values. The results show differences in CT values which were high for prostate cancer group.This study aimed to investigate the potential interaction of FOSL1 polymorphism on genetic damage and thyroid, breast and prostate cancer risk.Our findings are consistent as well with those displayed by [15]

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
Group 1 Healthy	23.125	1.093 E-7	1.09 E-7/1.09 E-7	1.000
Group 2 Prostatic Cancer	23.09	1.119 E-7	1.11 E-7/1.09 E-7	1.023
Group 3 Breast Cancer	23.11	1.104 E-7	1.09 E-7/1.09 E-7	1.010
Group 4 Thyroid Cancer	23.10	1.112 E-7	1.11 E-7/1.09 E-7	1.017

Their results indicate that the existence of a significant correlation between blood and tumor tissue expression of some genes of clinical interest, such as FOSL1 in gastric cancer, could allow the introduction in clinical practice of a simple test that would measure mRNA levels of DNA protein coding genes in peripheral blood samples instead of tissue samples to determine prognostic and predictive factors in thyroid, breast and prostate cancer patients.

In the present study, quantitative RT -PCR assay analyzed the mRNA expression of FOSL1 and compared its expression between apparently healthy control group, thyroid cancer group, breast cancer group and prostatic cancer group. The calculation of gene expression fold change was made using relative quantification.This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of fosl-1 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 Healthy group, prostate cancer group, breast cancer group and thyroid cancer group were (**-0.725**), (**-2.83**), (**-2.69**) and (**-2.42**) respectively. A significant difference was noticed between the study groups (p=0.001).

Results of 2-Ct revealed significantly higher results for the prostate cancer group from the other three groups (p=0.0001), mean of 2-Ct for prostate group (**7.110**). In the breast cancer group, thyroid cancer group and healthy group a mean of 2-Ct were (**6.453**) (**5.351**) (**1.641**), respectively.

To calculate the gene expression folds in relation to the housekeeping genes the result of $2^{-\Delta Ct}$ of each group was measured in relation to that of control group.Results are shown in Table (8). The fold of gene expression in prostatic cancer group was 4 time higher than that of healthy group. That for the breast cancer group was 3 times higher than the healthy group and the thyroid cancer group was 3 times higher than the healthy group. These results indicate significantly increase expression of FOSL1 gene in these groups.

Table (8) Fold of *FosL1*expression Depending on $2^{-\Delta Ct}$Method

Groups	Means Ct of *FosL1*	Means Ct of *GAPDH*	ΔCt (Means Ct of *FosL1* - Means Ct of *GAPDH*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 1 Healthy	22.41	23.125	-0.725	1.641	1.641/1.641	1.00
Group 2 Prostatic Cancer	20.26	23.09	-2.83	7.110	7.110/1.641	4.331
Group 3 Breast Cancer	20.42	23.11	-2.69	6.453	6.453/1.641	3.931
Group 4 Thyroid Cancer	20.68	23.10	-2.42	5.351	5.351/1.641	3.260

The calculation of the relative expression of FOSL1 gene in all study groups was done by applying the 2 -ΔΔCt results. A calibrator was used which was one of the samples of the controls with high expression of FOSL_1.As shown in (Table 10), the mean of 2-ΔΔCt values of Healthy group, prostate cancer group, breast cancer group and thyroid cancer group it was (**2.514**), (**10.890**), (**9.883**) and (**8.196**), respectively. There was a significant difference between these groups regarding the mean 2-ΔΔCt, (p=0.0001).

Table (9): Fold of *FosL1* expression Depending on 2-ΔΔCt Method

Groups	Means Ct of FosL1	Means Ct of GAPDH	ΔCt (Means Ct of FosL1	Mean ΔCt Calibrato	ΔΔCt	2-ΔΔCt	experimental group/ Control group	Fold of

			- Means Ct of GAPDH)	r (ct *FosL1*-ct *GAPDH*				ge ne ex pre ssi on
Group 1 Healthy	22.41	23.125	-0.725	0.615	-1.33	2.514	2.514/2.514	1.0 0
Group 2 Prostatic Cancer	20.26	23.09	-2.83	0.615	-3.445	10.890	10.890/2.53	4.3 31
Group 3 Breast Cancer	20.42	23.11	-2.69	0.615	-3.305	9.883	9.883/2.53	3.9 31
Group 4 Thyroid Cancer	20.68	23.10	-2.42	0.615	-3.035	8.196	8.196/2.53	3.2 60

Calculating gene expression was significantly higher in prostatic cancer group, 4 than. Fold number in breast cancer group was 3 times higher than the healthy group and the thyroid cancer group was 3 times higher than the healthy group shown in table (10) The above results demonstrate the significant gene expression in prostatic cancer group. 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.It is well shown here that the high expression was evident in prostatic cancer group in comparison to the healthy group. However, breast and thyroid cancer group also show a good number of high expressing individuals.Induction of FOSL1 gene expression is apparently partly a due to the cancer itself. There was a significant statistical differences between the study groups, $p<0.05$ suggesting the importance of detecting high expression of FOSL1 gene as a marker for DNA damage and repair, as an important conclusion of this experiment to be conducted on all cancer patients.

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