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

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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 FOSL1 in the peripheral blood was analyzed using reverse transcription polymerase chain reaction (RT-PCR). The expression of FOSL1 mRNA in the fold of gene expression in prostatic cancer group was 4 times 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.

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.

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.

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).

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
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manufacturer's instructions. The total RNA volume to be reversely transcribed was (20μl). Thermal cycler steps of cDNA Reverse Transcription are show in (Table 1).

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Step2</th>
<th>Time</th>
<th>Step3</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>10min</td>
<td>42°C</td>
<td>15min</td>
<td>85°C</td>
<td>5sec</td>
</tr>
<tr>
<td></td>
<td>Random Primer (N9)</td>
<td></td>
<td>Anchored Oligo(dT)18</td>
<td></td>
<td>Inactivate reverse transcriptase enzyme</td>
<td></td>
</tr>
</tbody>
</table>

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

Table (2): primers and probes

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5’→3’ direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FosL1 gene expr.</td>
<td>CACCCTAGCCAATGTCTCCT</td>
</tr>
<tr>
<td>Forward</td>
<td>AGAAACAGTGGGCAGCTTTG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAPDH house-keeping gene</td>
</tr>
<tr>
<td>DNA</td>
<td>TGAGAAGTATGTAGACAGCC</td>
</tr>
<tr>
<td>Nuclease free water (N.F.W)</td>
<td>TCTTCCAGATACAAAG</td>
</tr>
</tbody>
</table>

REAL TIME QRT-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.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix EasyTaq® PCR SuperMix</td>
<td>12.5μl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 μl</td>
</tr>
<tr>
<td>DNA</td>
<td>4μl</td>
</tr>
<tr>
<td>Nuclease free water (N.F.W)</td>
<td>6.5μl</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
<th>cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denaturation</td>
<td>95°C</td>
<td>5 m</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>95°C</td>
<td>30 S</td>
</tr>
<tr>
<td></td>
<td>annealing</td>
<td>58°C</td>
<td>40 S</td>
</tr>
<tr>
<td>3</td>
<td>Extension</td>
<td>72°C</td>
<td>40 S</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72°C</td>
<td>10 m</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>95°C</td>
<td>30 Sec</td>
<td>Hold</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>5 Sec</td>
<td></td>
</tr>
<tr>
<td>Anneal/extend</td>
<td>60°C</td>
<td>40 Sec</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>1min /95 °C-30 sec /55 °C-30sec/95 °C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

REAL TIME QRT-PCR ANALYSIS OF FOSL_1 GENES EXPRESSION

\[ \Delta Ct \]
The expression ratio was calculated without a calibrator sample $2^{-\Delta Ct}$ according to the following equation: 
$$2^{-\Delta Ct} = \text{Normalized expression ratio}$$

2. $\Delta \Delta CT$

To compare the transcript levels between different samples

$$\Delta CT = \text{target gene} - \text{internal control}$$

$$\Delta \Delta CT = \Delta CT_{text} - \Delta CT_{control}$$

The $\Delta \Delta CT$ of the test samples was normalized to the $\Delta CT$ of the calibrator:

$$\Delta \Delta CT = \Delta CT_{test} - \Delta CT_{calibrator}$$

Finally, the expression ratio was calculated according to the formula

$$2^{-\Delta \Delta Ct} = \text{Normalized expression ratio}.$$  

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 (23.1±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)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Mean ± SD of Ct value</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>25</td>
<td>23.125±0.05</td>
<td>23.09-23.2</td>
</tr>
<tr>
<td>Prostatic Cancer</td>
<td>25</td>
<td>23.09±0.06</td>
<td>23.05-23.23</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>25</td>
<td>23.11±0.05</td>
<td>23.01-23.14</td>
</tr>
<tr>
<td>Thyroid Cancer</td>
<td>25</td>
<td>23.1±0.07</td>
<td>23.04-23.22</td>
</tr>
<tr>
<td>LSD</td>
<td>2.644NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.732</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 $2^{-\Delta Ct}$ value and the ratio of the $2^\Delta Ct$ of the different study groups to that of control group, as shown in Table 7. The $2^{-\Delta Ct}$ 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.

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Their results indicate that the existence of a significant correlation between blood and tumor tissue expression of somatic 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-ΔCt 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 times 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-ΔCt Method**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Ct of FosL1</th>
<th>Mean Ct of GAPDH</th>
<th>ΔCt (Mean Ct of FosL1 - Mean Ct of GAPDH)</th>
<th>2-ΔCt</th>
<th>Experimental group/ Control group</th>
<th>Fold of gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Healthy</td>
<td>22.41</td>
<td>23.125</td>
<td>-0.725</td>
<td>1.641</td>
<td>1.641/1.641</td>
<td>1.00</td>
</tr>
<tr>
<td>Group 2 Prostatic</td>
<td>20.26</td>
<td>23.09</td>
<td>-2.83</td>
<td>7.110</td>
<td>7.110/1.641</td>
<td>4.331</td>
</tr>
<tr>
<td>Group 3 Breast</td>
<td>20.42</td>
<td>23.11</td>
<td>-2.69</td>
<td>6.453</td>
<td>6.453/1.641</td>
<td>3.931</td>
</tr>
<tr>
<td>Group 4 Thyroid</td>
<td>20.68</td>
<td>23.10</td>
<td>-2.42</td>
<td>5.351</td>
<td>5.351/1.641</td>
<td>3.260</td>
</tr>
</tbody>
</table>

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 FOSL1. 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**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Ct of FosL1</th>
<th>Mean Ct of GAPDH</th>
<th>ΔCt (Mean Ct of FosL1 - Mean Ct of GAPDH)</th>
<th>Mean ΔCt</th>
<th>ΔΔCt</th>
<th>2-ΔΔCt</th>
<th>Experimental group/ Control group</th>
<th>Fold of gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Healthy</td>
<td>22.41</td>
<td>23.125</td>
<td>-0.725</td>
<td>-2.42</td>
<td>5.351</td>
<td>5.351</td>
<td>1.641/1.641</td>
<td>1.00</td>
</tr>
<tr>
<td>Group 4 Thyroid</td>
<td>20.68</td>
<td>23.10</td>
<td>-2.42</td>
<td>5.351</td>
<td>5.351</td>
<td>5.351</td>
<td>1.641/1.641</td>
<td>3.260</td>
</tr>
</tbody>
</table>
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 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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