

# FOXA1 expression in Iraqi women with ER+ breast cancer

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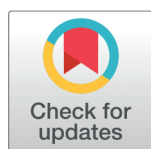
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## ABSTRACT

**Background:** Breast cancer (BC) is a heterogeneous disease that can be classified into many subtypes according to histopathological and molecular characteristics. Forkhead box protein A1 (FOX A1) is a transcriptional pioneer factor that opens chromatin allowing estrogen receptor ( $\alpha$ -ER) access to its genomic targets. FOXA1 expression is related to luminal BC with a good prognosis.

**Objectives:** The present study is sought to determine the FOXA1 expression in Iraqi women with ER+ BC.

**Methods:** Forty-eight fresh malignant breast tissues were analyzed by immunohistochemistry assay to choose ER+ samples, and then by RT-qPCR to evaluate FOXA1 gene expression.

**Results:** The ER-positive samples were (72.91%) of the total samples, and the molecular subtype of luminal A was the most common with a percentage of 56.25%. It was also noted that the high expression of the FOXA1 gene is highly significant ( $p < 0.05$ ) in Iraqi women with BC when compared with healthy controls.

**Conclusions:** Highly significant FOXA1 expression was found in Iraqi women with BC makes it eligible to be a good predictor or a biomarker for BC.

**Keywords** breast cancer, ER+, FOXA1, immunohistochemistry, RT-qPCR

## INTRODUCTION

Breast cancer (BC) is the most common malignant tumor ever been diagnosed and responsible for the second cancer-related mortality among women in Iraq, accounting for 23% of cancer-related deaths.<sup>1,2</sup> Estrogen receptor-alpha [ER- $\alpha$ ], which intermediates the action of estradiol in powering the development and progression of BC, is expressed in

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approximately 70% of the total malignant breast tumors that making estrogen receptor-positive (ER+) BC the most common BC subtype.<sup>3</sup> ER- $\alpha$  functions as an estrogen ligand-activated transcription factor to regulate various genes that govern cell proliferation and survival in normal tissues and tumors.<sup>4-6</sup>

Forkhead box A1 (FOXA1) is a forkhead family member protein encoded by the *FOXA1* gene located on chromosome 14q21.1.<sup>7,8</sup> FOXA1 is responsible for the postnatal development of mammary and prostate glands.<sup>9</sup> *FOXA1* expression is also associated with low breast tumor grade, exhibiting a positive correlation with the luminal A BC subtype.<sup>10</sup> Such observation suggests a strong correlation between *FOXA1* expression and luminal A breast tumor subtype; however, the co-regulatory partners of both molecules are still undefined. Some studies reported that FOXA1 and ER constitute a major proliferative and survival axis for BC.<sup>11</sup> FOXA1 is the archetypal pioneer factor, capable of binding to compact chromatin independently of other proteins and creating a localized euchromatic environment.<sup>12</sup> It can mediate ER binding events in BC cell lines.<sup>13-15</sup> It is required for the growth of drug-resistant cancer models,<sup>16</sup> and it has been shown to directly contribute to endocrine resistance.<sup>17,18</sup> *FOXA1* was primarily expressed in ER-positive BC metastases.<sup>19</sup> Because there are no data about the *FOXA1* gene expression in Iraqi BC population; thus, the present study was aimed to determine the gene expression of *FOXA1* in Iraqi women with ER+ BC from fresh tissue.

## MATERIALS AND METHODS

### Patients and sampling

Forty-eight fresh malignant breast tissues with surrounding normal tissues were collected during mastectomy from women with BC. Fresh tissue samples were collected from hospitals in Al-Najaf Al-Ashraf Governorate (Al-Sader Medical City, Bin Bilal Hospital, and Al Ghadeer Private Hospital), and Medical City of Baghdad between September 2018, to March 2019. Patients' ages were ranging from 27 to 91 years old. Each sample was divided into three parts: 1) malignant tissue part, 2) normal tissue part as control, and 3) malignant tissue part for immunohistochemistry assay. All parts were washed with sterilized normal saline and stored in RNAlater<sup>®</sup> solution (Sigma-Aldrich, Germany).

### Immunohistochemistry assay

In the current study, the immunostaining method used was the Labeled Strept-Avidin Biotin (LSAB+) technique which was applied for the staining of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) according to the manufacturer's protocol of DAKO company Dako, Carpintera, Ca, USA) w and Climent et al. (2001)<sup>20</sup>. Four  $\mu\text{m}$  thicknesses of the sections were cut, mounted into the silanized slides, then left to dry overnight at 37°C. All the sections were deparaffinized and

rehydrated. Antigen retrieval was achieved by heat retrieval using a bench autoclave. For a short time, the slides were placed in Coplin jars containing enough 0.01M sodium citrate solution (pH 6.0) to cover the sections, then autoclaved at (121°C) for (10 min) in the case of HER2 and (15 min) for both ER and PR. The slides were incubated with (100–200 $\mu$ l) of primary antibodies for (30 min) at room temperature in a moisture chamber then rinsed in phosphate-buffered saline (PBS). The dilution of the primary antibodies against ER and PR (Biogenex, San Ramon, CA, USA) was 1:130, and for HER2/neu (Dako, Carpinteria, Ca, USA) was 1:50. After washing, binding of antibodies was performed by incubation for (10 min) with biotinylated goat anti-mouse antibody ready to use (LSAB2) from Dako. The slides were then rinsed with PBS. And, sections were incubated with streptavidin-horse radish peroxidase for (10 min). Finally, the sections were washed 4 times for 4 min with PBS, followed by adding 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Germany) as a chromogen to produce the characteristic brown stain.

## RNA extraction and gene amplification with RT-PCR

TRIzol reagent (Invitrogen, Thermo Fisher, USA) was used to extract total RNA from the frozen samples preserved in RNAlater<sup>®</sup> solution. Total RNA was then reverse into cDNA using a AccuPower<sup>®</sup> Rocket Script<sup>™</sup> RT PreMix (BioNEER, Korea) according to the manufacturer's protocol. Briefly, 18 $\mu$ l of total RNA and 2 $\mu$ l oligo primer were added in a tube at 37°C for 10 min, 42°C for 50 min and then 95°C for 5 min. For quantitative or real-time polymerase chain reaction (qPCR or RT-PCR), mRNA levels were analyzed using SYBR-Green reagent (GoTaq<sup>®</sup> qPCR and RT-qPCR Systems, Promega, USA). Amplification conditions were as follows: Taq activation at 94°C for 2 min; 40 cycles of 94°C for 20 sec, 60°C for 1 min and elongation at 72°C for 30 sec. Gene expression of the target gene *FOXA1* and housekeeping gene *GAPDH* were analyzed using specific primers for each one. For *GAPDH* were: F- 5'ATCACTGCCACCCAGAAGACTG3' and R- 5'AGGTTTTTCTAGACGGCAGGTCAG 3', and for *FOXA1* were F- 5'GTT GAA GAC TCC AGC CTC CTC3' and R- 5'CTG CCC AGA ACA TCA TCC CT3'. A  $2^{-\Delta\Delta C_t}$  was used to determine the differences in the gene expression because it is a reliable method.<sup>21</sup> RT-PCR was performed in duplicate for each sample; then, mean values were used at the final calculation for mRNA expression level.

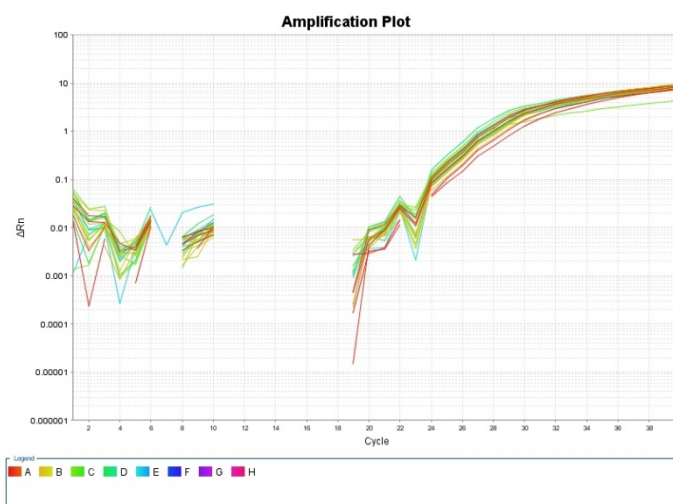
## RNA concentration and purity

TRIzol<sup>®</sup> was used for the isolation of total RNA from 30 tumor tissue samples, only those who showed a strong positive ER in the immunohistochemistry results, and 30 normal tissue samples preserved in RNALater<sup>®</sup> which preserved the tissues as a fresh tissue without any change. Total RNA concentration was determined by a non-traditional method with Qubit<sup>®</sup> RNA HS assay kits (Thermo Fisher, USA), and the RNA concentration range was

from low to high concentration between (4.7-46.1 ng/ $\mu$ l).

## Quantitative real-time polymerase chain reaction (qPCR)

RT-nested PCR was used to optimize *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase, a common housekeeping gene for sample normalization and widely used in the qRT-PCR) forward and reverse primers; all the concentrations were given optimal reaction and appeared as similar peaks as shown in Figure 1.



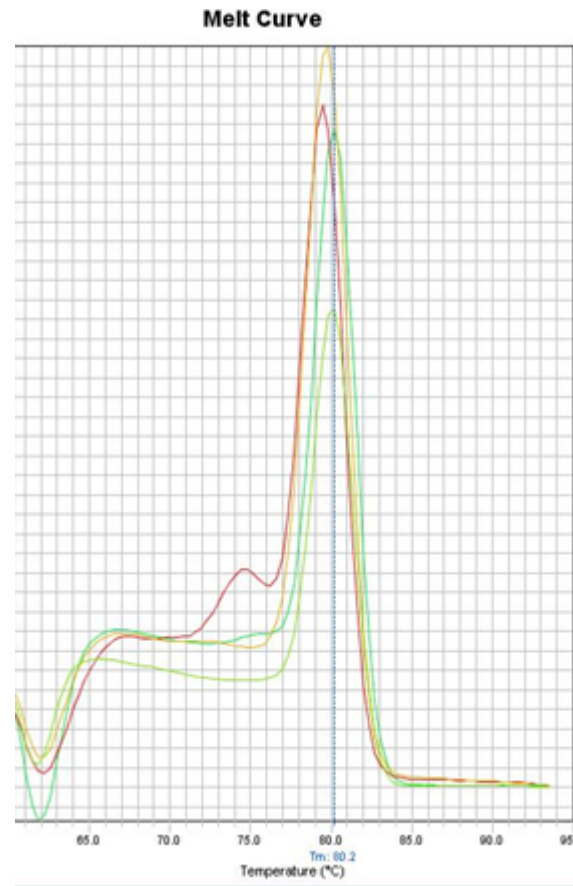
**Figure 1** The optimization of different concentrations (Nested method) for *GAPDH* forward and reverse primers in qPCR.

## Optimization of *FOXA1* primers' temperature

The optimal annealing temperature was detected after applying three degrees closed to the manufacturer's recommendation of 60°C. The perfect peaks appear at the same recommended temperature for three primers. The melting curve showed distinct single peaks ( $T_m$ = 77-80°C), Figure 2. The photograph was taken directly from Applied Biosystems 7500 qPCR.

## RESULTS AND DISCUSSION

The current study included 48 BC patients. Among them, 45/48 (93.75%) were married, 11/48 (22.91%) were with family history either of 1<sup>st</sup> or 2<sup>nd</sup> degree, 45/48 (93.75%) with



**Figure 2** Melting temperature ranged from 79°C to 81°C, no primer dimer could be seen.

ductal carcinoma, 35/48 (72.91%) in grade II and 25/48 (52.08%) with left lateral breast carcinoma. The median patients' age was 50 years with a rang between 27-91 years.

The statistical analysis of data (Table 1) showed a significant ( $p \leq 0.01$ ) differences between studied clinicopathological features except in BC location, which revealed no statistically significance difference between cancer if left or right breast.

The present results on Iraqi women patients revealed that a high percentage of BC occurred between 41-49 (33.33%) and 51-60 (31.25%) years of age, respectively; which indicates that BC is more frequent in these age groups than others (Table 2).

In a recent study in Iraq BC by Al-Alwan et al. (2019),<sup>17</sup> the prevalence of BC in age classes 20-34, 35-49, 50-64 and  $\geq 65$  was 4.4%, 42.4%, 42.2 and 11%, respectively. Also, these results are in agreement with other Iraqi studies such as that of Al-Nuaimy et al., (2016)<sup>16</sup> who mentioned that 36.7% and 29% of women's BC occurred at ages between 40-49 and 50-59 years, respectively. Jumaah (2013) also reported a high frequency (38.89%) of BC occurred in ages between 51-60 years.<sup>22</sup> In another study by Majid et al. (2017)<sup>18</sup>, the results revealed a significant increased BC rate between 2006 and 2012 among women  $\geq 60$  years old ( $p < 0.001$ ).

**Table 1** Clinicopathological data in the patients with breast cancer.

Clinic-pathological features	No(%)
<b>Family history</b>	
Yes	11(24%)
No	37(76%)
<b>Marital status</b>	
Married	45(93.75%)
Non-married	3(6.25%)
<b>Breast type</b>	
Ductal	45(93.75%)
Lobular	3(6.25%)
<b>Grade</b>	
II	35(72.92%)
III	13(27.08%)
<b>Location</b>	
Lateral, right	23(47.92%)
Lateral, left	25(52.09%)

**Table 2** Distribution of sample study according to age groups.

Age group (year)	No. of patient
≤40	10
41-50	16
51-60	15
>60	7
<b>Total</b>	<b>48</b>
<b>Chi-square (<math>\chi^2</math>)</b>	<b>6.935</b>
<b>P</b>	<b>≤0.01</b>

## Immunohistochemistry results (ER, PR, and HER2)

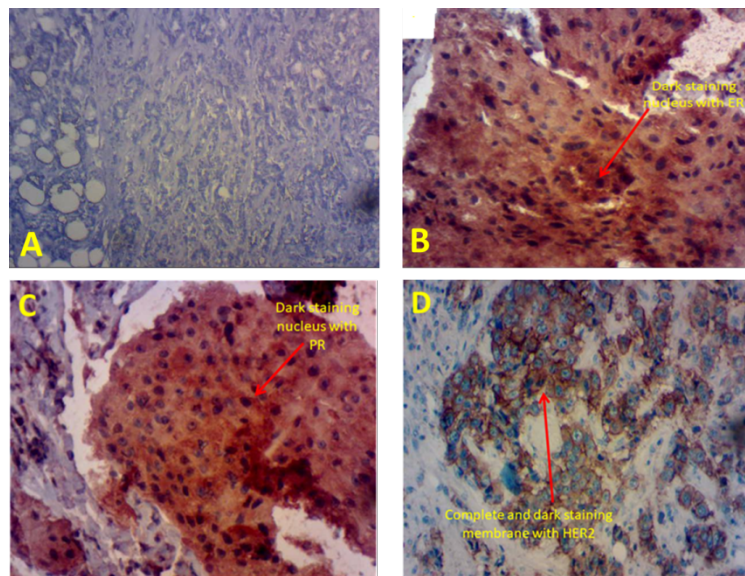
ER, PR and HER2 were biogenic factors and very important in the early stage of BC.<sup>23</sup> Tumour showing positive receptors has a better prognosis and better response to hormonal therapy than those with no receptors.<sup>24</sup>

**Table 3** Hormonal results of ER, PR and HER2 by immunohistochemistry method.

Hormone	No. of positive	%	No. of negative	%
ER	35/48	72.92	13/48	27.08
PR	27/48	56.25	21/48	43.75
HER2	11/48	22.91	37/48	77.08

Immunohistochemical profiles of 48 BC samples (Table 3, and Figure 3) showed that ER receptors were positive in 72.91% (35/48) of the cases and PR positive receptors in 56.25% (27/48) of the cases, which indicates that there is a hormone receptor expression in the majority of studied BC cases. The present results were also demonstrated that 11(22.9%)

out of 48 malignant cases were positive for HER2 expression, while 37 cases out of 48 were with score 0 and score 1 and 6 cases with score 2 considered (equivocal) as HER2 negative result. These results appear to be inline with that reported rates of 20% to 30% by Almasri et al. (2005),<sup>25</sup> Azizun-Nisa et al. (2008),<sup>26</sup> and Mudduwa (2009)<sup>27</sup> but higher than that completely reported by Cho and his colleagues in 2008<sup>28</sup>. Our findings were similar to those of many studies such as Xu et al. (2018)<sup>29</sup> who found: ER+ was (38/53) 71.70%, ER- was (15/53) 28.30%, PR+ was (35/53) 66.04%, PR- (18/53) 33.96%, HER2+ was (9/53) 16.98%, HER- (44/53) 83.02%. Also, the results were nearby with the results were gained by Ma et al. (2018),<sup>30</sup> who mentioned that the Luminal-like BC (ER+ and/or PR+) was 73.9% and ER2+ was 7.6%.



**Figure 3** Representative staining results from H&E and IHC (A): Negative staining for ER, PR, HER2 in malignant breast cell magnification 10X (score 0). B): ER staining in metastatic breast cancer, magnification 40X (score 3). C): PR staining in metastatic breast cancer, magnification 40X (score 3). D): HER-2 protein (score 3) low power 10X.

### Quantification of *GAPDH* expression (using RT-PCR)

The *Ct* value of *GAPDH* is shown in Table 4. The range in BC patients was (18.44-37.02) with a mean $\pm$ SE of (28.91 $\pm$ 0.93). While, in controls, it was (23.05- 33.71) with a mean $\pm$ SE of (27.45 $\pm$ 0.64).

### Quantification of *FOXA1* expression (using RT-PCR)

The results of gene expression for *FOXA1* (Figure 4) in the present study is shown in Table 5. The range of expression for the *FOXA1* gene in BC patients was (0.02-6.35) with

**Table 4** Comparison of *GAPDH* (housekeeping gene) expression in Iraqi patients and controls.

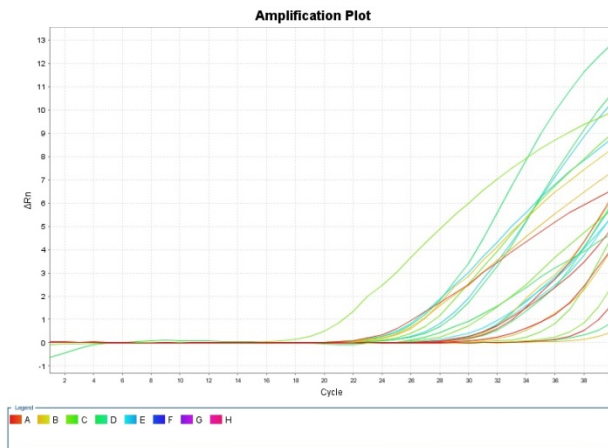
Group	No.	Mean±SE
Patients	30	28.91±0.93
Control	30	27.45±0.64
<i>t</i> -test	–	2.264
<i>P</i> -value	–	0.203 <sup>NS</sup>

NS: Non-significant

a mean±SD of (1.097±0.24) while in the control group, it's ranging from (0.0002-0.0364) with a mean±SD (0.0079±0.002). There was a significant difference seen between the BC patients and control group regarding the mean *Ct* value of *FOXA1* ( $p=0.0001$ ) with a *t*-value of (4.490,  $p\leq 0.01$ ).

**Table 5** *FOXA1* gene expression in Iraqi patients and controls.

Group	No.	Mean±SE
Patients	30	1.097±0.24
Control	30	0.0079±0.002
<i>t</i> -test	–	<b>4.490</b>
<i>P</i> -value	–	<b>0.001</b>



**Figure 4** *FOXA1* amplification plots in qPCR appeared *Ct* values. The photograph was taken directly from Applied Biosystems 7500 qPCR.

Table 6 illustrates *FOXA1* expression in BC patients and controls (it was high when the change fold above 1, and low when the change fold less than 1). These results showed much higher expression in cancerous tissues than in controls, and highly significant low expression in controls versus in patients with a *p*-value of 0.0001.

Our results are entirely inline with those of many studies that investigated *FOXA1* expression in BC and others diseases. A meta-analysis for the prognostic value of *FOXA1* in

**Table 6** Comparison of fold values of *FOX A1* expression between patients and control

Group	High expression % ( $\leq 1$ )	Low expression% ( $> 1$ )
Patient	14 (46.7) %	16(53.3 %)
Control	0 (0.0%)	30(100%)
<b>P -value</b>	<b>0.0001</b>	

BC which was conducted by Shou et al. (2016),<sup>31</sup> included nine articles comprising 6386 BC patients. The results suggested that high *FOX A1* expression was positively associated with BC. The approximation of our results appeared obviously with two Japanese studies done by Hisamatsu et al. (2012)<sup>32</sup> and Hisamatsu et al. (2015)<sup>33</sup> were confirmed that *FOX A1* expression was positively associated with ER in BC patients ( $p < 0.0001$ ). *FOX A1*, a hormone-receptor-positive BC driver, harbors a mutational hotspot in its promoter that leads to over expression through increased E2F binding, leading to BC. These findings, which observed by Rheinbay et al. (2017),<sup>34</sup> agreed with our results and interpreted why the high expression of *FOX A1* gene in Iraqi women with ER+ BC. In another meta-analysis by Chinese authors, including seven studies have identified the association between *FOX A1* expression and the ER+ in BC, the study proven that *FOX A1* expression level was higher in the ER+ BC group when compared with the ER- BC group.<sup>35</sup> Also, Wang et al. (2019)<sup>36</sup> mentioned that the expression of *FOX A1* is associated with the prognosis of estrogen receptor (ER+) in BC. Similarly, an american study by Badve et al. (2007)<sup>37</sup> mentioned that *FOX A1* expression was seen in 75% (300 of 404) of BC patients, and it's correlated with ER+ with ( $p = 0.000001$ ). As well, in another Chinese study by Dai et al. (2019),<sup>38</sup> the authors found high levels of *FOX A1* in 68.75% of luminal BC patients. Fu et al. (2016)<sup>15</sup> were also found that *FOX A1* and ER+ co-expressed at high levels in metastases of BC that were resistant to endocrine therapy. The same results obtained by De Lara et al. (2019),<sup>13</sup> Wang et al. (2018),<sup>39</sup> and Michailidou et al. (2018),<sup>40</sup>. In 2019, Jiang et al. explained that *FOX A1* associate with stronger ER- $\alpha$  binding by (co-operative pre-loading) on more nearby chromatin containing ER- $\alpha$  motifs.<sup>41</sup> *FOX A1* was identified as a transcriptional regulator in some liver-specific genes. It is expressed in different tumors, the most important is BC and could bind to the promoters of many hundred genes associated with regulation of cell signalling in addition to the cell cycle, including estrogen. However, *FOX A1* role is unclear yet, some of the studies reported that *FOX A1* and ER- $\alpha$  constitute a major proliferative and survival axis for BC, and its expression correlates with a favorable outcome in BC.<sup>9 12 42 43</sup> In addition to its role in other cancers, like prostate cancer, when Yang and Yu (2015)<sup>44</sup> mentioned that *FOX A1* regulates prostatic and non-prostatic androgen-chromatin targeting. There was a present an updated model wherein FOXA1: AR equilibrium in the nuclei defined prostatic androgen binding profile; that was perturbed in prostate cancer with *FOX A1* and/or androgen deregulation in addition to (Song et al., 2019).<sup>45</sup>

The major limitations of the current study are the relatively small-sized group of patients and the limited diversity (i.e. most of them were from the Middle Euphrates region). However, this is the first study about FOXA1 expression in Iraqi women with ER+ BC.

## CONCLUSIONS

Our finding regarding the high expression level of *FOXA1* in the first Iraqi study) BC patients with a high percentage that corroborate and confirm that FOXA1 have a strong prognostic role in BC (tumorigenesis) and could be conceder a significant predictor (prognostic biomarker) in primary BC with ER-positive that may be proved right decisions in clinical treatment.

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## DECLARATIONS

### Authors' contributions

Conceptualization: IHA & AMA. Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Validation, Visualization, Writing-original draft: IHA. Methodology: IHA, KD, MM. Software: IHA, KD, MM. Supervision: AMA. Writing-review & editing: IHA & AMA. All authors have reviewed and approved the final version of this article before publication.

### Conflict of interest

There are no conflicts of interest to report.

### Ethical approvals

This project has been reviewed and approved by the scientific committee of the Genetic Engineering and Biotechnology Institute, University of Baghdad, and the Laboratories Department of the Iraqi Health Ministry on August 15, 2018. Written informed consent was obtained from each participant before participation in the current study.

### Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

### Funding resources

This work didn't receive any fund.

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