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Gene expression analysis of FGFR2 in breast cancer patients using real-time qPCR

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ABSTRACT

Background. *Fibroblast growth factor receptor 2 (FGFR2) is a protein involved in numerous biological processes, including embryonic development, angiogenesis, tissue maintenance, and cancer progression.*

Aim of the study. *To evaluate FGFR2 mRNA expression levels in breast cancer patients and assess its potential as a diagnostic biomarker using RT-qPCR.*

Methodology. *Fifty blood samples were analyzed (25 from breast cancer patients and 25 from healthy controls). Two milliliters of each specimen were used for extraction of ribonucleic acid (RNA) and synthesis of complementary DNA (cDNA) from the extracted RNA, followed by evaluation of FGFR2 gene expression using RT-qPCR. The 2−ΔΔCt method was used for relative quantification of FGFR2 mRNA expression, normalized to the β-actin reference gene.*

Results. *FGFR2 gene expression analysis demonstrated a significant 7.5-fold increase in expression ($p < 0.05$) in the breast cancer group compared with the control group.*

Conclusion. *FGFR2 gene expression was significantly higher in breast cancer patients than in healthy controls, suggesting that FGFR2 may represent a potential non-invasive biomarker for breast cancer. Further studies with larger cohorts and protein-level validation are needed.*

Keywords: FGFR2, breast cancer, RT-qPCR, gene expression, whole blood, biomarker

INTRODUCTION

Fibroblast growth factor receptors (FGFRs) play pivotal roles in various biological processes, including regulatory signaling, tissue growth, differentiation, migration, and cellular function. FGFR2 is a member of the FGFR family and contributes to the regulation of cell division, movement, and development through interaction with fibroblast growth factors and other signaling molecules [1]. The signaling pathways activated by FGFRs are also important in numerous physiological functions in the adult body, such as the regulation of blood vessel formation and tissue repair [2]. FGFRs are found in a variety of cell types and regulate essential cellular behaviors in cancer cells [3]. In the early stages of bone development, fibroblast growth factor receptors 1 and 2 regulate osteogenesis and are also found in the perichondrium and periosteum [4]. Conversely, FGFR3 is primarily present in chondrocytes and has been shown to influence the ossification process by regulating bone tissue formation [5].

Among the FGFRs, FGFR2 has a clear role in bone development. Recent findings have shown that dysregulation of FGFR2 signaling is commonly observed in different types of malignancies and hematologic disorders [6]. Additional biological alterations in FGFR3 include gene amplification in urinary tumors and mutations in various plasma cell cancers [7]. Moreover, disruption of fibroblast growth factor signaling has been documented in multiple cancer types. FGFR1 expression has been observed in approximately 10% of breast cancers and oral squamous cell carcinomas, and it has also been identified at lower rates in ovarian cancer, bladder cancer, and rhabdomyosarcoma [8]. FGFR2 mutations can

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be found in 12% of uterine tumors, while they are apparently infrequent in gastrointestinal tumors [9]. High levels of FGFR2 expression and molecular alterations have also been reported in gastrointestinal malignancies [10]. Targeted therapy generally relies on diagnostic testing that demonstrates overexpression or molecular alteration of the FGFR2 gene in tumor tissue [11]. However, FGFR2 expression may not always be detectable in diagnostic biopsies at the initial tumor stage, and tumor evolution, as well as variable patterns of FGFR2 expression, may influence the efficacy of selected treatments, further supporting the exploration of FGFR2 as a therapeutic target in gastrointestinal tumors [12].

FGFR2 is required for appropriate embryonic patterning, trophoblastic activity, limb bud formation, lung development, bone formation, and skin development. It plays a crucial role in controlling osteoblast differentiation, growth, and apoptosis and is necessary for normal skeletal development. It also promotes cell growth in keratinocytes and immature osteoblasts [13].

The thermocycler technique was originally used to identify specific nucleotide sequences and has developed into a reliable research instrument in biological and medical fields. Its application to RNA research was based on the use of reverse transcription to generate a DNA template, followed by amplification through polymerase chain reaction, a process referred to as reverse transcription polymerase chain reaction (RT-PCR) [14]. However, due to limitations in precise quantification, standard PCR underwent refinements that led to the development of a more powerful analytical technique known as real-time quantitative PCR (RT-qPCR) [15].

RT-qPCR is a versatile technique with applications ranging from fundamental molecular research to genetic identification and molecular diagnostics [16]. Its broader use is particularly relevant in clinical and research settings, including in developing countries [17]. This technique modifies the conventional PCR approach by enabling real-time monitoring of the amplification process [18]. RT-qPCR is an enzyme-based laboratory method used to exponentially amplify and quantify specific nucleic acid targets [19]. DNA template, primers, nucleotides (dNTPs), and heat-stable DNA polymerase are required as reaction components [20].

The current study aimed to evaluate FGFR2 gene expression and assess its potential as a biomarker in breast cancer using RT-qPCR.

MATERIALS AND METHODS

Study design and participants

The patient group (n = 25) consisted of female breast cancer patients aged 20 to 60 years who had not received prior chemotherapy. The control group (n = 25) comprised healthy females of the same age group (Table 1).

TABLE 1. *Demographic and clinical characteristics of study participants*

Characteristic	Patient Group (n = 25)	Control Group (n = 25)
Age (years), Mean ± SD	45.2 ± 8.5*	46.1 ± 7.9*
Age range (years)	20-60	20-60
Sex	All Female	All Female
Diagnosis	Breast Cancer	Healthy
Prior chemotherapy	No	N/A

In total, 50 blood samples were collected for FGFR2 analysis from a private laboratory in Basrah between March and May 2024. The participants' ages ranged from 20 to 60 years. In the control group, 25 blood samples were collected using a sterile syringe from healthy volunteers. Two milliliters of each sample were placed into EDTA tubes and processed within 2 hours to ensure RNA stability for RNA extraction in both groups. Subsequently, cDNA was synthesized from all RNA samples, and SYBR Green was used to measure FGFR2 (target gene) and β-actin (housekeeping gene) expression levels using RT-qPCR.

The selection of β-actin as a single reference gene was justified by its established stability in breast cancer research and whole blood samples. Previous studies on breast cancer progression and RT-qPCR normalization, including research on Iraqi women with breast cancer, support the use of β-actin as an internal control for data normalization in this study [21-23].

Gene expression analysis of FGFR2 gene

RNA was extracted from whole blood according to the manufacturer's guidelines using the GENEzol™ TriRNA Pure kit (Geneaid). All extraction steps were carried out at 4°C as outlined below. A total of 200 µL of blood was added to a 1.5 mL microcentrifuge tube, followed by the addition of 700 µL of GENEzol™ reagent, and then allowed to stand at room temperature for 5 minutes. The solution was centrifuged at 12,000 rpm for 1 minute, and the clear supernatant was transferred into a sterile 1.5 mL microcentrifuge tube. Absolute ethanol of an equal volume was added and mixed thoroughly using a vortex.

A total of 700 µL of the mixture was transferred to the RB column in a 2 mL collection tube and centrifuged at 12,000 rpm for 1 minute; the flow-through was discarded. The remaining mixture underwent the same process. The column was then placed into a new collection tube. A total of 400 µL of wash buffer was added and centrifuged at 12,000 rpm for 30 seconds.

Five microliters of DNase were mixed with 45 µL of DNase buffer for each sample, added to the center of

the RB column, and incubated at 25°C for 15 minutes. Then, 400 µL of pre-wash buffer was added and centrifuged at 12,000 rpm for 30 seconds, after which the flow-through was discarded. This step was repeated twice using wash buffer. The RB column was centrifuged at 12,000 rpm for 3 minutes to remove residual moisture and transferred into a clean, sterile 1.5 mL microcentrifuge tube.

The isolated RNA was eluted by adding 50 µL of RNase-free water to the center of the column, incubating it for 2 minutes, and centrifuging at 12,000 rpm for 1 minute. RNA purity and concentration were assessed using a Nanodrop spectrophotometer, and the A260/A280 ratio was determined after preparing the blank with RNase-free water. RNA samples were stored at –80°C for further analysis.

cDNA synthesis for RT-PCR

Total RNA from each sample was converted into cDNA using the AccuPower® RocketScript™ RT PreMix kit (Table 2). RNA template and nuclease-free water were added to the cDNA master mix tubes, mixed thoroughly using a mini vortex, and subjected to thermal cycling according to the conditions shown in Table 3.

TABLE 2. *Reference volumes for a single cDNA reaction*

Reagents	Volume (µl)
RNA template	2
Nuclease free water	18
Total volume	20

TABLE 3. *cDNA synthesis program for RT-PCR*

Steps	Temperature	Time
Primer annealing	37 °C	10 min
cDNA synthesis	60 °C	1 hr
Heat inactivation	95 °C	5 min

Table 4 shows the FGFR2 primer sequences and their length [24]. Table 5 presents the reaction volumes for RT-qPCR using SYBR Green, while Table 6 shows the FGFR2 gene expression protocol.

RT-qPCR quality control and statistical analysis

To ensure the reliability and accuracy of the RT-qPCR results, several quality control measures were implemented. All samples were analyzed in technical triplicates, and consistency of Ct values across replicates was verified. No-template controls (NTCs) were included in each run to detect potential contamination, and all NTCs yielded negative results.

Specificity of the amplification products was confirmed by melt curve analysis, which showed a single distinct peak for both FGFR2 and β-actin, indicating the absence of primer-dimers or non-specific amplification. Furthermore, to control for potential genomic DNA contamination, all RNA samples underwent DNase I treatment before cDNA synthesis.

TABLE 4. *Primer used for amplify FGFR2 gene and their length*

No.	Primer	Primers sequence	Primer length
1	FGFR2-F	5'-GCTGACTTCTATTTATATAACTTCAAGC-3'	28
2	FGFR2-R	5'-CAGAAGTTTTTGAGAGTGGCATGATC-3'	26

TABLE 5. *Volumes for a single qPCR reaction using SYBR Green*

Reagents	Volume (µl)
Go Taq RT-qPCR	8
F primer	2
R primer	2
cDNA	1
Double distilled water	7
Total amount	20

TABLE 6. *FGFR2 gene expression protocol*

Stages	Temperature (°C)	Time	Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	45
Annealing	60°C	30 sec	
Extension	72°C	40 sec	

Both ΔCt and ΔΔCt values were calculated for individual samples in the patient and control groups to represent biological variability. ΔCt values were statistically compared using Student's t-test, as they represent normalized expression levels and typically follow a normal distribution. Normality of ΔCt values was tested using GraphPad Prism 9 ($p > 0.05$). A two-tailed t-test was applied, and equality of variances was assessed using the F-test.

For β-actin, the primer sequences used were forward 5'-ATGGGTCAGAAGGATTCCTATGT-3' and reverse 5'-AGCCACACGCAGCTCATT-3' [25]. PCR reactions were carried out in a total volume of 20 µL, as described in Table 5 [26].

RESULTS

Estimating of expression level of the FGFR2 gene

Melt curve analysis confirmed the specificity of the RT-qPCR products, showing a single, sharp peak for both FGFR2 and β-actin, indicating the absence of non-specific amplification or primer-dimers. The distinct melting temperatures (Tm) for each amplicon further validated the purity of the PCR products (Figure 1).

For the FGFR2 gene, primers labeled with the green fluorescent dye SYBR were used as the detection system, and primer binding was specific to the target gene (Figure 2). Relative mRNA expression levels and the comparison between breast cancer patients and healthy controls are illustrated in Figure 3.

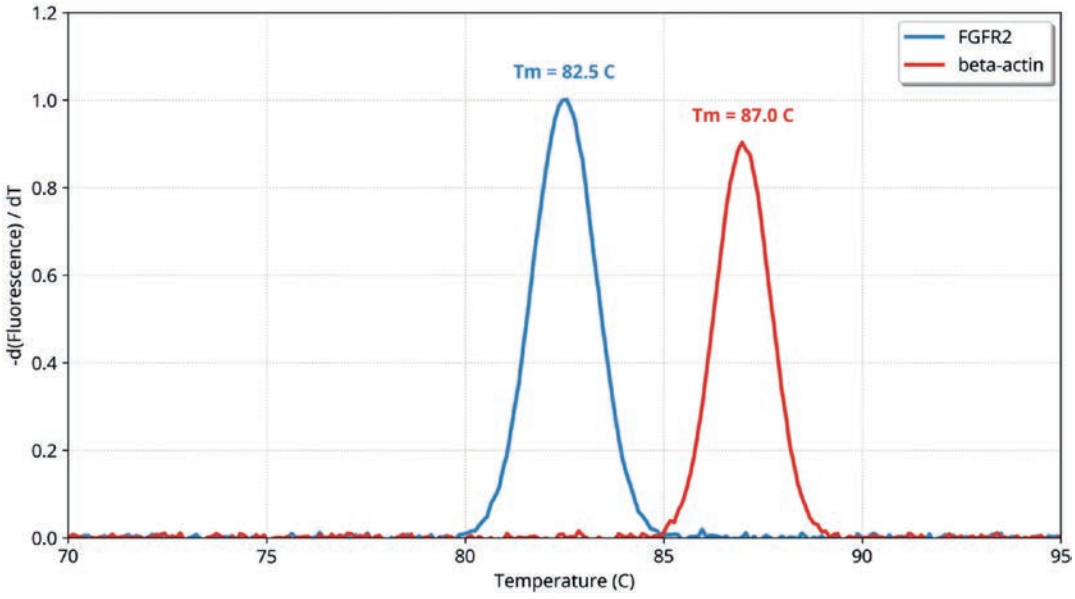

FIGURE 1. *Melt curve analysis of FGFR2 and β-actin amplification products*

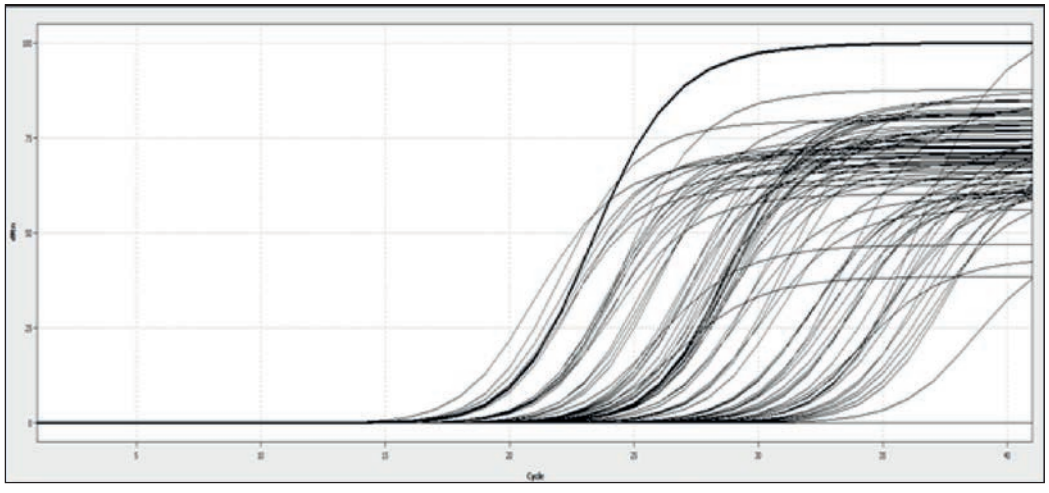

FIGURE 2. *Real-time RT-qPCR amplification plots for the FGFR2 gene*

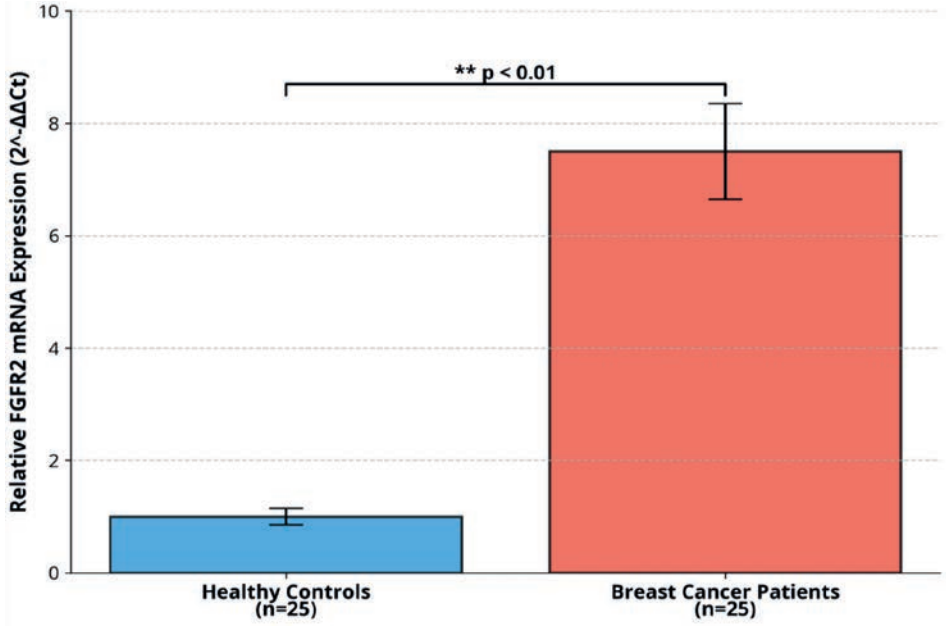

FIGURE 3. *Relative mRNA expression of the FGFR2 gene in breast cancer patients and healthy controls*

TABLE 7. *Quantitative results of FGFR2 mRNA expression levels in breast cancer patients and healthy controls*

Study group	Sample size (N)	Mean Ct (FGFR2) ± SD	Mean Ct (β-actin) ± SD	Mean ΔCt±SD	Fold change (2–ΔΔCt)	p-value
Breast cancer patients	25	24.12 ± 1.15	18.34 ± 0.85	5.78 ± 0.42	7.5	< 0.01
Healthy controls	25	26.85 ± 1.42	18.15 ± 0.92	8.70 ± 0.65	1.0 (calibrator)	-

The RT-qPCR results were analyzed using the ΔΔCt method. Cycle threshold (Ct) values for the FGFR2 gene and the housekeeping gene (β-actin) were determined [25]. The following steps were used to calculate gene expression [27]:

ΔCt = Ct target gene – Ct housekeeping gene

ΔCt (patient) = Ct patient – Ct housekeeping gene

ΔCt (control) = Ct control – Ct housekeeping gene

ΔΔCt = ΔCt patient – ΔCt control

Gene expression = 2-ΔΔCT

Fold change (FC) = expression (patients) / expression (controls)

As shown in Table 7, quantitative analysis using the 2−ΔΔCt method revealed a significant 7.5-fold increase in FGFR2 mRNA expression in the breast cancer patient group compared with the healthy control group ($p < 0.05$). The β-actin gene was used as an internal reference for normalization.

The stability of β-actin expression was confirmed within the dataset, with mean Ct values of 18.34 ± 0.85 in the patient group and 18.52 ± 0.92 in the control group, showing no statistically significant difference ($p = 0.48$).

DISCUSSION

Gene amplification and overexpression of fibroblast growth factor receptor 2 (FGFR2) have been identified as important features in cancer biology and targeted therapies, as reported by Katoh et al. [28]. In the present study, we demonstrated a significant 7.5-fold increase in FGFR2 mRNA expression in breast cancer patients compared with healthy controls, supporting its potential involvement in tumorigenesis. These findings are consistent with previous reports indicating increased FGFR2 expression in malignant tissues and its association with tumor progression [29-31].

The observed overexpression of FGFR2 may reflect its role in promoting cellular proliferation, survival, and angiogenesis, processes that are critical in cancer development. FGFR2 signaling has been implicated in pathways involved in tumor growth and progression, as supported by previous studies on fibroblast growth factor receptor biology and oncogenic signaling [3,4,32,33]. Similar observations of elevated protein and mRNA expression levels have been reported in various tumor types [30,31]. This biological relevance supports the potential of FGFR2 as both a diagnostic biomarker and a therapeutic target.

The use of β-actin as a housekeeping gene provided stable normalization in this study, which is consistent with previous reports supporting its application in gene expression studies [21,22]. The absence of significant variation in β-actin expression between groups strengthens the reliability of the relative quantification results obtained using the 2−ΔΔCt method.

RT-qPCR is a sensitive and specific technique for gene expression analysis and was appropriately used in this study [11,12]. It allows accurate quantification of nucleic acids and reduces the need for post-amplification processing, thereby minimizing analytical errors. These advantages have contributed to its widespread use in molecular diagnostics and gene expression studies [16,17].

The role of fibroblast growth factors (FGFs) in tissue development, cellular differentiation, and bone formation has been well documented [18,19,34-40]. FGFR2 specifically plays a crucial role in cellular growth and regulation, supporting its involvement in cancer-related processes [3,4,41,42].

Accurate quantification of RNA in clinical samples depends on normalization of the target gene against housekeeping genes [43]. RT-qPCR is increasingly used alongside conventional techniques for gene expression analysis [44]. The results obtained from RT-PCR are often complemented by real-time PCR for improved quantification [45,46]. Housekeeping genes are generally expressed consistently across tissues; however, their expression should be stable and not influenced by disease conditions [47,48].

Despite these findings, the clinical applicability of FGFR2 as a biomarker requires further investigation. Although overexpression was clearly demonstrated, additional studies are needed to determine its diagnostic sensitivity and specificity, as well as its prognostic value and response to targeted therapies.

Limitations

The limitations of this study include the relatively small sample size and the focus on mRNA expression levels only. Further studies are required to validate these findings at the protein level using techniques such as Western blotting.

CONCLUSION

FGFR2 gene expression was significantly higher in breast cancer patients than in healthy controls, sug-

gesting that FGFR2 may represent a potential non-invasive biomarker for breast cancer. However, the present study evaluated mRNA expression only and did not assess protein expression or clinical outcomes. Further studies with larger cohorts and protein-level validation are needed to confirm the diagnostic and prognostic value of FGFR2 in breast cancer.

Ethical approval

The Ethics Committee of the College of Science, University of Basrah, approved the study on March 5, 2024 (Approval No. EC/358). Written informed consent was obtained before blood collection in accordance with the Declaration of Helsinki.

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Conflict of interest

The authors declare no conflict of interest.

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Authors' contributions

Conceptualization, Hala F. Hassan, Adnan I. Al-Badran; methodology, Hala F. Hassan; software, Adnan I. Al-Badran; validation, Hala F. Hassan; formal analysis, Adnan I. Al-Badran; investigation, Adnan I. Al-Badran; resources, Hala F. Hassan; data curation, Hala F. Hassan; writing—original draft preparation, Adnan I. Al-Badran; writing—review and editing, Hala F. Hassan; visualization, Hala F. Hassan; supervision, Adnan I. Al-Badran; project administration, Hala F. Hassan; funding acquisition, Adnan I. Al-Badran. All authors have read and agreed to the published version of the manuscript.

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