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Original Article

Investigation of Interferon Gamma Activity Using Bioinformatics Methods

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Abstract

Breast cancer grows from the breast tissue and is a severe health problem worldwide. Genetics is believed to be the primary cause of all cases of breast cancer via gene mutation. Bioinformatics methodology has been used to determine the sequences and structures of bioactive substances. This study aimed to analyze the function and structure of the Interferon Gamma (IFNγ) in healthy controls and patients with breast cancer using bioinformatics methods. Blood samples were collected from 75 patients with breast cancer and 25 healthy subjects as control samples. The results showed transition mutation (30%) and transversion mutation (70%) in patients with breast cancer. Moreover, missense mutations (84%) and silent mutations (16%) were detected by BLAST. In addition, the amino acid of the IFNγ protein consisting of alpha-helical, β-sheet, and coil of secondary structure was determined in this study using BioEdit. The results of the physicochemical properties of the IFNγ protein reflect the function, stability, molecular weight, isoelectric point, and instability index of the IFNγ protein using ProtParam. Moreover, the results of mutation affected the percentage of alpha-helix, β-turns, and coil in breast cancer patients compared to healthy groups with reference of NCBI using PSIpred program. Additionally, the PHYRE2 server and RasMol program showed a tertiary structure of the IFNγ protein in breast cancer patients. Furthermore, the STRING program revealed the poly IFNγ protein interacted with other proteins to perform its functions normally. From the recorded data in the current study, it was concluded that IFNγ is considered a marker for patients with breast cancer.

Keywords: Interferon Gamma, Breast Cancer, Bioinformatics Technique

1. Introduction

Breast cancer grows from the breast tissue and is a severe health problem worldwide. Genetics is believed to be the primary cause of all cases of breast cancer via gene mutation (1). Interferon-gamma (*IFNγ*) is a dimer protein and a sole cytokine of the interferon type II family. The signaling of the *IFNγ* requires a complex receptor IFNGRs and the JAK-STAT system. The cellular responses to *IFNγ* are activated through its interaction with a heterodimeric receptor consisting of interferon-gamma receptor 1 (IFNGR1) and interferon-gamma receptor 2 (IFNGR2). The *IFNγ* binding to the receptor activates the JAK-STAT pathway (2). The JAK is Janus kinases but the STAT is a signal transducer and activator of transcription proteins (3). The STAT is phosphorylated by JAK which leads to forming gamma activated factor. This factor is found in the nucleus and binds with a specific sequence of DNA leading to modulating the *IFNγ* target gene transcription in the cell. This pathway in normal cases is called a STAT-dependent manner. However, in abnormal cases, it is called a STAT-independent manner, and the *IFNγ* signaling is stimulated via some genes leading to activate NF-κB to regulate gene expression (4). Some IFN-target genes have also been

associated with chemotherapy resistance that induces apoptosis and cell cycle arrest (5). Some studies revealed that the *IFNγ* signaling was detected in breast cancer cells (6). The JAK-STAT signaling in mammary glands located within breasts can promote cell division and reduce cell apoptosis during normal cases; however, in abnormal cases, during cancer formation, it leads to excessive activation or disruption of JAK-STAT signaling (7). The JAK-STAT signaling pathway is a chain of interactions among proteins in a cell and is involved in processes, such as immunity, cell division, cell death, and tumor formation. The STATs are made by protein biosynthesis that called post-translational modifications. These modifications include methylation, acetylation, and serine phosphorylation. The serine phosphorylation is required for the transcription of some target genes of the cytokines, such as IFN-γ (8). Nevertheless, a limited number of studies have focused on the molecular mechanisms of the *IFNγ* genes in breast cancer cells (9).

This study used the bioinformatics methods as modern molecular biology to reveal the mutations in the *IFNγ* sequence and translate the sequence of the *IFNγ*. Similarly, molecular methods were utilized to detect the physiochemical properties of the *IFNγ* gene and determine the protein function. These methods were applied in mammary tumors and healthy samples.

2. Materials and Methods

2.1. Collection of Samples

The blood samples were collected from 100 Iraqi patients (25 control and 75 patients) with breast cancer, aged between 20 and 70 years. These samples were collected from AL-Amal National Hospital for Cancer Treatment in Baghdad Province, Iraq, from October 2020 to January 2021. The blood samples were stored frozen at 4°C until use.

2.2. Primers Used in Conventional PCR

The primers used for sequencing the *IFNγ* are listed in table 1.

Table 1. Sequence of specific primers for the *IFNγ* gene

Primer	Sequence	Product size
Generic Primer	5'-TCAACAAAGCTGATACTCCA- 3'	261 bp
Specific A	5'-TTCTTACAACACAAAATCAAATCA - 3'	261 bp

2.3. DNA Extraction

DNA extraction from the blood samples was used for the purification of DNA from the whole blood (serum or plasma) using an extraction kit.

2.4. PCR Program

The PCR program is illustrated in table 2.

Table 2. Optimum condition for the detection of the *IFNγ* gene

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	95°C	1 min	1 cycle
2	Denaturation-2	95°C	20 sec	30 cycle
3	Annealing	62°C	1 min	30 cycle
4	Extension-1	72°C	50 sec	30 cycle
5	Extension-2	72°C	5 min	1 cycle

2.5. To Confirm PCR Products

To confirm the PCR products, agarose gel (2%) was made using Tris-Borate EDTA (TBE) buffer (Conda/USA). Subsequently, the DNA samples along with SiZer DNA (plus 1000) were loaded, and the electrophoresis was run at f 7 v\cm for 1-2 h to separate the DNA products already stained with RedSafe Nucleic Acid Stain (Intron/Korea). Eventually, the DNA bands were visualized using a UV transilluminator.

2.6. Sequencing the Product of Amplification of the *IFNγ* Gene

DNA was sent to Korea (Macrogen) for sequencing using the Sanger method.

2.7. Bioinformatics Analysis of the *IFNγ* Gene

Bioinformatics analysis of the *IFNγ* gene using several programs is as below:

a- BLAST Program was used for determining the position of mutations in the *IFNγ* sequence and comparing with the reference sequence of the *IFNγ* gene in NCBI

b- BioEdit Program Translation Sequence Tool was used to translate the *IFNγ* gene sequences to amino acid sequences

c- ProtParam Program was used to detect the physiochemical properties of the *IFNγ* gene

d- PSIpred Online Software was used to confirm the *IFNγ* protein

e- Phyre2 Program was used to predict and analyze protein structure, function, and mutations

f- STRING Program was used to determine the protein function

3. Results

3.1. Results of Conventional PCR for the Detection of Extracted DNA of the *IFNγ* Gene

Gel electrophoresis revealed the bands of extracted nucleic acid from the *IFNγ* gene of healthy samples and patients with breast cancer (Figure 1).

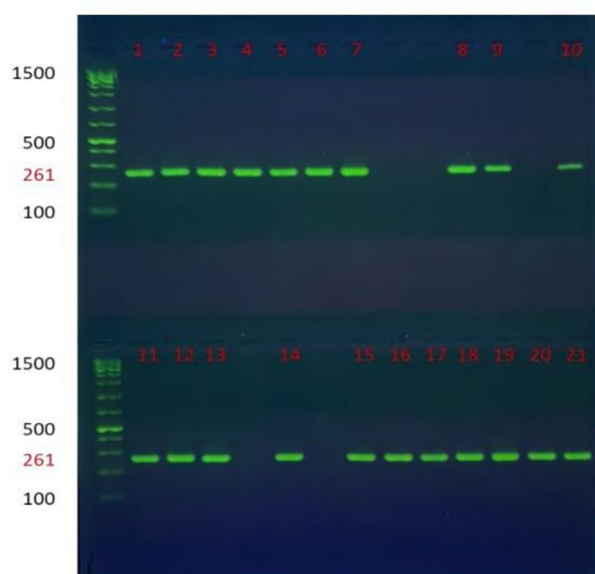

Figure 1. Agarose gel electrophoresis pattern showing the conventional PCR for the *IFNγ* gene in infected and non-infected samples

3.2. Results of Sequencing of the *IFNγ* Gene Using the BLAST Program

Sequence analysis of the PCR products for the *IFNγ* gene showed that four substitution transition type mutations appeared in 30% of the total samples. This occurred as follows: Thymine substitution by Cytosine at locations 6069, 6095, 6098, 6170, and 6208 (T>C), while Adenine substitution by Guanine was found at locations 6034, 6046, 6054, 6096, 6224, 6227, and 6230 (A>G), and finally Cytosine substitution was observed by Thymine at the location 6232 (C>T). On the other hand, 11 substitution transversion type mutations appeared in 70% of the samples as follows: Adenine substitution by Cytosine at locations 6026, 6032, 6042, 6046, 6064, 6096, and 6230 (A>C), as well as Adenine substitution by Thymine at locations 6022, 6026, 6087, 6122, 6125, 6225, and 6230 (A>T).

The results for the second primer at the exon 4 region (coding exon) are for breast cancer patients, and the point mutations were found including transition and transversion substitution mutations. Missense mutation changed the Wild genetic code of the amino acid Arginine (CGU) to be another amino acid Proline (CCU) by substituting Guanine to cytosine (G→C). Furthermore, the genetic code of the Phenylalanine (UUU) was mutated to be the genetic code of Leucine (UUA) by substituting uracil to adenine (U→A). The analysis of the sequences of the *IFNγ* gene for the breast cancer patients showed many genetic variations, and the types of the appeared mutations are listed in tables 3 and 4.

Table 3. Effect of mutations on the protein and their percentage at DNA of the *IFNγ* gene in breast cancer patients

Type of mutation	Mutation percentage (%)
Transition	30%
Transversion	70%

Table 4. Type of mutations and their percentage at DNA of the IFNγ gene in breast cancer patients

Effect of the translation	Percentage (%)
Missense	84%
Silent	16%

3.3. Results of Translation of Amino Acid from Sequencing of the *IFNγ* Gene Using BioEdit Program Translation Sequence Tool

The result of the translation of amino acids from the sequencing of the *IFNγ* gene is illustrated in

figure (2), and it is composed of alpha-helix, β-sheet, and coil for two different patients A and B. For patient A, alpha helix, β-turns, and coil prediction values were 37.56%, 34.91%, and 27.53%, respectively. Furthermore, considering patient B, alpha helix, β-turns, and coil prediction values were obtained at 36.97%, 24.42%, and 38.61%, respectively.

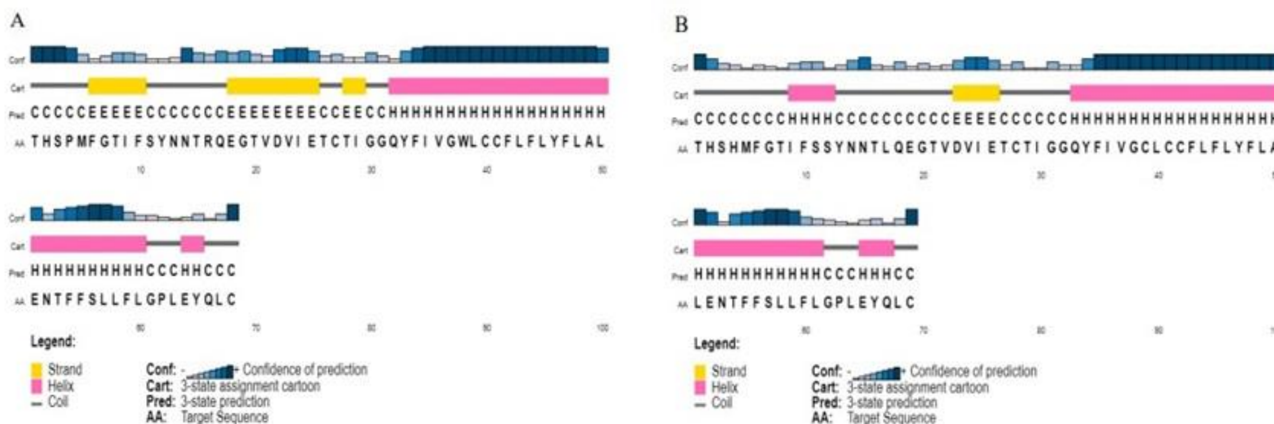

Figure 2. Confirmation of the *IFNγ* protein using the BioEdit software for drawing structure protein (helixes, β-sheets, and coils) of amino acids

3.4. Results of the Physicochemical Properties of the *IFNγ* Gene Using ProtParam Program

Table 5 tabulates the results of the physicochemical properties of the human *IFNγ* protein for healthy control and cancer groups. These properties reflect the function, stability, effect of the protein, and many other features. These physicochemical properties, including molecular weight, Isoelectric Point (PI), and Instability Index (II) are listed in table 5.

Table 5. Physiochemical prosperities of the *IFNγ* protein for the breast cancer patients and healthy controls with the reference human *IFNγ* of NCBI

Group	Sample	M.Wt.	PI	Instability Index(II)
Ref Seq	-	19348.30	9.50	30.29
Breast cancer	1.	5149.88	5.43	69.71
Breast cancer	2.	5479.96	5.47	42.45
Breast cancer	3.	10229.05	5.43	39.04
Breast cancer	4.	5361.83	5.71	37.66
Breast cancer	5.	5161.88	5.47	90.73
Breast cancer	6.	5286.11	5.43	99.31
Breast cancer	7.	5177.49	5.43	90.57
Breast cancer	8.	4751.10	5.52	42.78
Breast cancer	9.	4945.47	5.71	54.29
Breast cancer	10.	4947.32	5.51	45.14
Breast cancer	11.	4945.74	5.46	54.29
Breast cancer	12.	4977.53	5.46	55.71
Breast cancer	13.	5141.44	5.48	34.76
Breast cancer	14.	4632.97	5.52	44.29
Breast cancer	15.	5103.80	5.44	67.83
Healthy control	1.	4714.14	5.47	44.67
Healthy control	2.	5311.74	5.17	49.23
Healthy control	3.	5009.65	5.45	71.77
Healthy control	4.	1430.52	5.51	10.88
Healthy control	5.	4975.65	5.49	60.50
Healthy control	6.	4797.12	5.48	34.05
Healthy control	7.	2588.74	5.18	52.01
Healthy control	8.	3378.59	5.50	29.46
Healthy control	9.	2672.88	5.18	59.44
Healthy control	10.	2947.14	5.50	22.56
Healthy control	11.	4783.10	5.48	36.10
Healthy control	12.	5119.79	5.44	75.15
Healthy control	13	3323.57	5.18	56.81

3.5. Results of Protein Conformation of the *IFNγ* Gene Using the PSIpred Online Software

The results showed that the mutation affected the percentage of alpha-helix, β-turns, and random coil in breast cancer patients and healthy groups, compared to the reference of NCBI. These percentages are illustrated in table 6.

Table 6. Percentages of alpha-helix, β-strand, and the random coil of the secondary structure of the *IFNγ* protein in the breast cancer patients, compared to the healthy control and the reference of NCBI

Group	Sample No.	A-Helix%	ß-Strand %	COIL %
Breast Cancer Patients	1	37.56	34.91	27.53
	2	36.97	24.42	38.61
	3	31.14	28.04	40.82
	4	32.43	31.41	36.16
	5	39.23	26.12	3465
	6	38.88	19.34	41.78
	7	33.52	25.54	40.94
	8	39.67	24.02	36.31
	9	38.19	19.92	41.89
	10	29.32	17.14	53.54
	11	32.17	18.75	49.08
	12	35.06	30.21	34.73
	13	46.11	27.67	26.22
	14	25.18	38.24	36.58
	15	29.97	29.34	40.69
Healthy Control	1	35.09	25.69	39.22
	2	38.12	35.32	26.56
	3	38.93	26.27	34.80
Refrence	1	25.45	34.98	39.57

3.6. Results of the Tertiary Structure of the *IFNγ* Protein Using PHYRE2 Server and RasMol Program

The results of the tertiary structure of the *IFNγ* protein in the breast cancer patients that were predicted with multiple shapes using PHYRE2 server and RasMol program are shown in figures 3 and 4, receptively.

3.7. Results of the Function of the *IFNγ* Protein Using STRING Program

The results showed that the *IFNγ* protein interacted with other proteins to perform its functions normally. This appeared in some samples of breast cancer patients, as well as the healthy Iraqi population as shown in figure 5.

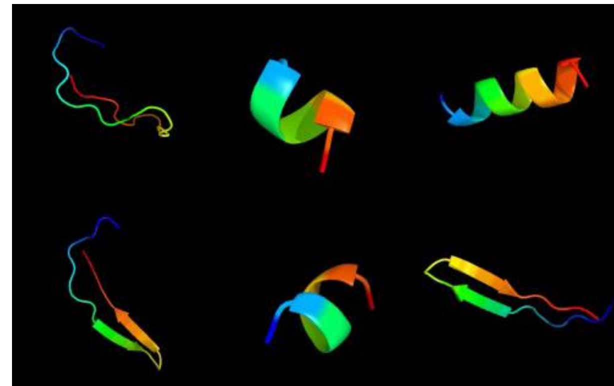

Figure 3. *IFNγ* tertiary structure prediction modeling using PHYRE2 server illustrating the confidence in the model along its length. The colors from high (red) to low (blue) use a rainbow spectrum (from N→C terminus)

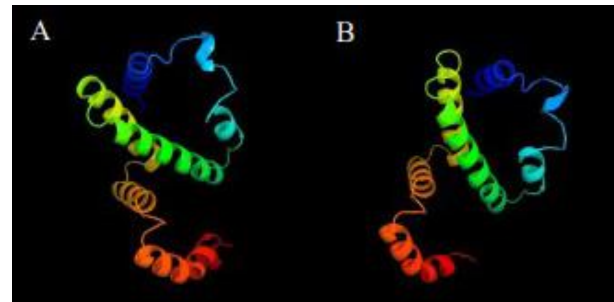

Figure 4. *IFNγ* tertiary structure prediction modeling using PHYRE2 server showing healthy control subjects **(A)**, and the *IFNγ* retrieved from NCBI **(B)** illustrating the confidence in the model along its length. The colors from high (red) to low (blue) use a rainbow spectrum (from N→C terminus)

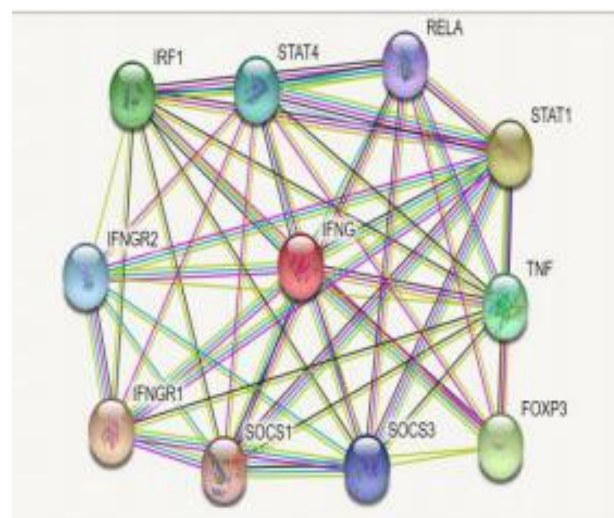

Figure 5. Network interaction of the *IFNγ* (red color) with other proteins

4. Discussion

Breast cancer is a severe health problem worldwide that grows within the mammary gland. In mammary healthy glands, the JAK-STAT-dependent pathway reduces cell apoptosis (2). However, in mammary tumor glands, it leads to extreme activation and disturbance in the JAK-STAT-independent pathway. The IFN γ is activated through the JAK-STAT system (4, 6). The stimulation of the *IFNγ* in breast cancer cells leads to morphological changes. These changes modulate the gene expression and may up-regulate and down-regulate the genes (10). The results of the breast cancer patients revealed 4 substitution transition type mutations in 30% and 11 substitution transversion type mutations in 70% of the patients. The results revealed that the point mutation was included the transition and transversion substitution mutations. The transition mutations occur when one pyrimidine base replaces another pyrimidine base or when one purine base replaces another purine base. However, the transversion refers to a DNA point mutation in which a single (two rings) purine is replaced with a (one ring) pyrimidine or vice versa (11).

Moreover, the analysis of the *IFNγ* gene in breast cancer patients found missense and silent mutations in 84% and 16% of the cases, respectively. A missense mutation is a mistake in the DNA that causes the incorrect amino acid to be incorporated into a protein (12). On the other hand, silent mutations are DNA mutations that have no discernible effect on the organism's phenotype (13-15). These mutations were found in the same locations as in the healthy control and were not found in the IFN gene of the reference sequence that indicates the genetic variations in the polymorphism of the Iraqi population (16, 17). The results of point mutations in the exon that influenced the amino acid changes had effects on the type, stability, and properties; moreover, it caused alteration in the entire function of the protein; therefore, its activity led to diseases (18). In contrast, the mutations that originate in intron are considered important mutations due to an association with some types of cancer (breast cancer) (19-22). Moreover, any changes in the nucleotide sequence of a gene affect the structure and function of the protein. This is clearly demonstrated by various effects on the physicochemical properties, as well as the structures of the produced protein. The results of mutations on the IFN gene detected in this study demonstrated the same effects and were consistent with the findings of the previous studies (23, 24).

The results of the primary structure of the protein provide the physiochemical properties of the human *IFNγ* protein for healthy control and cancer groups. These properties reflect the function, stability, effect of the protein, and many other features (24). The results of the physicochemical properties in the structure of the *IFNγ* protein due to the substitution of amino acids affect the synthesized protein and its function (25). The molecular weight results of human *IFNγ* for patients with breast cancer were decreased or increased, compared to that in healthy subjects. The II is one of the primary protein structure-dependent methods available for *in vivo* protein stability predictions in which proteins with II value below 40 are stable proteins. While above 40 is unstable protein. All samples were stable but in different ranges. The PI of the protein was the least stable. The result of PI less than 7 means precipitation in acidic buffers, while the result of PI greater than 7 means the solubility is in basic buffers (26, 27). The results of the current study are in line with the former testing of the mutation effect on the primary structure of the protein (28). The results of the secondary proteins of the *IFNγ* protein in breast cancer patients demonstrated the mutation effect on alpha-helix, β-turns, and coil (29-31).

In this study, the *IFNγ* gene mutations had effects on the secondary and tertiary structure of the *IFNγ* protein that was consistent with the findings of some studies (24, 32, 33). Several studies revealed that mutation in the nucleotide sequence can affect the structures of the produced protein. The mutation that changes the amino acid arginine to lysine in breast cancer patients disturbs the alpha helix structure and is considered one of the

disease-causing mutations (34). The results showed the types of mutations and the structural changes of the protein which were predicted using bioinformatics tools that were in line with the results of (32, 35).

They demonstrated that changes in the gene can cause different types of diseases, including cancer, which can be detected easily using bioinformatics tools to make efforts for the early detection of diseases, as well as finding the proper medication easily. Similarly, the results of the STRING program showed that the *IFNγ* protein interacted with other proteins to perform its functions normally. The interaction of the *IFNγ* include IFNGR1 and IFNGR2 which are associated with IFNGR2 and IFNGR1, respectively, to form a receptor for the cytokine *IFNγ* and STAT1 that mediates the cellular responses to IFNs, cytokine, and other growth factors. STAT4 carries out a dual function: signal transduction and activation of transcription. However, the interaction with SOCS1 and SOCS3 regulates cytokine signal transduction. Moreover, the interaction with FOXP3 is important for the development and inhibitory functions of regulatory T cells. IRF1 displays a remarkable functional diversity in the regulation of cellular responses, and TNF induces the cell death of certain tumor cell lines. RELA is the endpoint of a series of signal transduction events and is related to many biological processes, such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis.

These findings are in line with the results of (36) and demonstrate that mutations to a protein's function cause a change in the specificity of interactions between a mutated protein and molecules that interact with it specifically, such as other proteins and nucleic acids. As a result, there is either a loss of function or a gain of function. Our findings are consistent with the results of (37-39) and demonstrate the importance of using bioinformatics to reveal the effect of mutations on protein structure, which affects the stability and function of the produced protein.

In conclusion, this study aimed to investigate the potential of the *IFNγ* as a marker for breast cancer using different bioinformatics techniques. Firstly, based on the obtained results of mutations (transition type), the *IFNγ* gene implicated breast cancer. These mutations were considered the polymorphism features for breast cancer patients and healthy subjects of the Iraqi population. Furthermore, the *IFNγ* gene mutations affect the function of the *IFNγ* protein in breast cancer patients according to the STRING program. Future studies should be conducted to predict how the mutation of the *IFNγ* can be associated with other types of diseases.

Authors' Contribution

Study concept and design: N. E. H.
Acquisition of data: A. A. A.
Analysis and interpretation of data: N. E. H.
Drafting of the manuscript: A. A. A.
Critical revision of the manuscript for important intellectual content: N. E. H.
Statistical analysis: A. A. A.
Administrative, technical, and material support: N. E. H.

Ethics

All the procedures were approved by the Ethics Committee at the University of Technology, Baghdad, Iraq under the project number (No. 2020-5478-5471).

Conflict of Interest

The authors declare that they have no conflict of interest.

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