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Article

Role of IL-21 Gene Expiration Association with Breast Cancer in Iraqi Patents

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Abstract:
Interleukin 21 (IL21) is a cytokine produced predominantly by a cluster of differentiation 4 (CD4+) T-cells and natural killer T-cells. In the present study, we demonstrated differential expression of IL21 in breast cancer samples using reverse transcription-polymerase chain reaction (RT-PCR). Breast cancer occurs due to mutations, or abnormal changes in breast cells, in the genes responsible for regulating the growth of cells and keeping them healthy. The genes are in each cell's nucleus, which acts as the "control room" of each cell. Typically, the cells in our bodies replace themselves through an orderly process of cell growth: healthy new cells take over as old ones die out. But over time, mutations can "turn on" specific genes and "turn off" others in a cell. That changed cell gains the ability to keep dividing without control or order, producing more cells just like it and forming a tumor. In this study, we intended to detect the expiration of the Interleukin 21 (IL-21) gene in breast cancer patients and its influence on breast cancer tumor size metastasis status and tumor grade by collecting fifty peripheral blood samples from breast cancer patients diagnosed with breast cancer and follow up in Al-Amal hospital and Al-Andalus privet hospital and the sample collection extended from /2022 to /2022 and twenty-Three healthy look volunteers as a control group. The blood samples collected from patients and healthy volunteers were used to extract RNA, and the molecular method RT-PCR using a specific primer for the IL-21 gene was used. The result shows that there was overexpression in the patient sample compared with healthy volunteers, and we found overexpression in metastasis cases of the patient rather than no metastasis. Also, we found that patients with a T4 state have IL-21 overexpression compared to other patients' tumor sizes.
Keywords: Breast cancer, interleukin 21, Tumor size, Metastasis, Grade

INTRODUCTION

Breast cancer is a highly heterogeneous disease due to its diverse morphological features, the variable clinical outcome and the response to different therapeutic options. It is therefore necessary to devise a clinically meaningful classification of the disease, which has to be scientifically sound, clinically proper and widely reproducible. The established histopathological classification has limited clinical utility due to insufficient prognostic and predictive power 1. Breast cancer is divided histologically into two main types, ductal lobular types, and divided according to molecular receptors of the cancerous cells into (Luminal A, Luminal B, Triple Negative Breast cancer, and Enriched Her 2) 2. Breast cancer is divided into different stages depending on Tumor size, lymph node involvement, and metastasis, as shown in table [1,2,3].

T	Primary tumor size
T0	No evidence for primary tumor
Tis	Carcinoma in situ
T1	Tumor of 2 cm or less in greatest dimension
T2	Tumor larger than 2 cm, but not more than 5 cm in greatest dimension
T3	Tumor larger than 5 cm in greatest dimension
T4	Tumor of any size with direct extension to chest wall of skin
N	Regional lymph node involvement
N0	No regional lymph node metastases
N1	Metastases in movable ipsilateral axillary lymph node(s)
N2: - Metastases in fixed ipsilateral axillary lymph node(s) or in clinically apparent ipsi-lateral internal mammary lymph node(s) in the absence of clinically evident axillary lymph node involvement	
N3: - Metastases in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement or in clinically apparent ipsilateral internal mammary lymph node(s) in the presence of clinically evident axillary lymph node metastases	
M	Presence of distant metastases
M0	No distant metastases
M1	Distant métastases

Table 1: Classification of breast tumor according to tumor size, lymph node involvement, and metastasis stat the size of the primary tumor (T), involvement of regional lymph nodes (N), and the presence of distant metastases (M).

Grade	Differentiation status Total score
I	Well differentiated 3-5 points
II	Moderately differentiated 6-7 points
III	Poorly differentiated 8-9 points

Table 2. Grading classification 3.

Interleukin 21 (IL-21), which belongs to the common γ-chain (γc) family, is a novel cytokine affecting T-cell proliferation, survival, and function [4]. In the case of cancer studies, the roles of IL-21, either as a tumor suppressor or oncogene, have been discussed in many previous studies. For example, IL-21 could induce the anti-tumor effects of NK cells via the NKG2D pathway [5]. Additionally, IL-21 enhanced NK cell lytic activity against Ab-coated tumor cells IL-21 activates cytotoxic programs in both $CD8^+$ T and NK cells, thus providing potent cytotoxic effector arms against cancer cells [6]. In breast cancer, it was observed that IL-21 might serve as an oncogene through The expression of IL21R in solid tumors and was involved in signaling pathways of matrix metalloproteinases (MMPs) that are crucial for the spreading and migration of metastatic MDA231 cells.[7].

MATERIALS AND METHODS

Study Design & Subjects.

Fifty Iraqi Women patients with breast cancer who attended Al-Amal Hospital and Al-Andalus Specialist Hospital during the period extended from 1 December 2021 to 23 February 2022, with ages ranging from 30 – to 67 years, were registered in this study. The required information about the patients and the histopathological properties of the tumors were recorded from the patient's files. All of the patients were diagnosed. These patients were from different stages of the disease and different age groups (30 – 67 years). All the cases were subjected to molecular study. The samples were preserved with TRIzol.

Twenty-three healthy volunteers with ages ranging from 26 – to 63 years; all twenty-five women were unrelated with no family history of breast cancer.

Blood sample collection

Venous blood was taken from patients and healthy volunteers (Control group) at about two milliliters (ml). These samples were placed in EDTA tubes.

Molecular detection

RNA extraction and purification

Sample lysis

for each tube, 0.4 ml of blood was added to 0.6 ml of Trizol reagent, then homogenized by pipetting up and down several times.

Sample purification.

For each tube, 130 µl of chloroform is added and then mixed by vortex for 15 seconds.

Transfer the tubes to the freezer for 15 minutes.

Then centrifuge for 15 minutes at 13000 RPM

Transfer the supernatant to a new tube for the next step.

RNA precipitations

For each tube, 300 µl isopropanol is added to the supernatant and then mixed by vortex for 15 seconds.

Transfer the tubes to the freezer for 25 minutes.

Then centrifuge for 10 minutes at 13000 RPM

Remove the supernatant and keep the pellet for the next step.

RNA washing

For each tube, 600 µl of 75% ethanol is added to the pellet and then mixed by vortex for 3 seconds.

Then centrifuge for 5 minutes at 13000 RPM

Remove the supernatant and let the pellet dry by air.

RNA solubility

The pellet is rehydrated by adding 25 µl of nuclease-free water.

RNA Concentration

Quantus Fluorometer is used to detect the extracted RNA concentration to measure the sample quantity for the next application. One µl of RNA sample was added to 199 µl of diluted dye and mixed. Then incubated at room temperature for 5 minutes in a dark place. After incubation, the RNA concentration was detected by Quantus Fluorometer.

Quantitative Real-time PCR (RT-qPCR)

Primer design preparation

The sequence of (the IL-21) gene obtained from the NCBI gene bank primer was designed with melting temperatures ranging from 60-62 C°. The primer length was 22 nucleotides primers supplied by Macrogen company and shown in Table 3

Primer	Sequence (5`→3`)	AnnealingTemp. (C°)
IL-21 Forward	CCAAGGTCAAGATCGCCACATG	60
IL-21 Reverse	TGGAGCTGGCAGAAATTCAGGG	
TBP Forward	CAGTCTGCGAATGGTACTAA	
TBP Reverse	TCAGTGGGGGTGAATTCAGTG	

Table 3. shows the primers used in RT-qPCR of the target gene (IL-21) and the Housekeeping gene (TBP)

The primers were supplied in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of (100 pmol/µl) as a stock solution, and then the working solution was prepared by adding 10 µl from the stock solution in the new tube and then adding 90 µl of nuclease-free water

One step Quantitative Real-time PCR Assay (QRT-PCR) protocol IL-21 genes

Quantitative real-time PCR (QRT-PCR) technology has recently reached a level of sensitivity, accuracy, and practical ease that supports its use as a routine bio instrumentation for gene level measurement. Several applications have already been implemented in cancer research, and others are being validated, showing that this molecular biology tool can provide researchers and clinicians with precious information concerning the behavior of tumors [8]. In this experiment, the (QRT-PCR) protocol is done as follows: Table 4 and Table 5.

Master mix component	Volume for 1 sample
Q PCR master mix	10.0 µl
RT mix	0.6 µl
MgCl2	0.3 µl
CXR	0.4 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Nuclease free water	6.7 µl
RNA	5.0 µl
Total volume	25.0 µl
Aliquot for singe rxn	20 µl of master mix in a tube, add 5 µl of RNA templet

Table 4. Reaction steps in One step qRT-PCR protocol

Step	Temperature Cº	Time	Cycle
RT enzyme activation	37	15:00	1
RT enzyme inactivation	95	10:00	
Denaturation	95	00:10	60
Annealing	60	00:30	
Extension	72	00:30	

Table 5. Program of one-step QRT-PCR

Statically analysis

The static analysis was done using Microsoft Excel 2016. The one-way analysis of variance (ANOVA) is used to determine whether there are statistically significant differences between groups. Then, we calculate the less significant differences (LSD) to know the differences between the groups. A p-value less than 0.05 was considered statistically significant [9].

RESULT

Fold IL-21 Patients	**2.870910873**
Fold IL-21 Control	**1.056842323**
Degree of freedom *of*	**71**
P-value	**0.000561692**

Table 6. Comparison of fold expression between patents and control group

In this study, we found that the IL-21 gene expression is significantly increased in the T4 group than in other groups of tumor size T1, T2, and T3, illustrated in Table 7. This result agreed with [10] studies thatshowing significant associations between increasing the expression and the increase of tumor size.

Groups of tumor size	Fold expression of IL-21.
T1	**1.344248524**
T2	**3.068552263**
T3	**2.788310529**
T4	**5.399217269**
Degree of freedom *of*	**46**
P-value	**0.024**
LSD	**1.98**

Table 7. Comparison of fold expression of IL-21 between tumor size groups

Group	Fold expression of IL-21.
Metastasis	**5.581886782**
None Metastasis	**2.0798837**
Degree of freedom *of*	**41**
P-value	**0.00000142**

Table 8. Comparison of fold expression of IL-21 between metastasis and none metastasis

Also, we found no statistically significant associations with the grading group, which is shown in Table 9 and agrees with the result presented by [10].

Groups of grading	Fold expression of IL-21.
Grade 1	**1.308039473**
Grade 2	**2.995996057**
Grade 3	**3.691175666**
Degree of freedom *of*	**47**
P-value	**0.127**

Table 9. Comparison of fold expression of IL-21 between grading groups

DISCUSSION

This study involved 60 cases of breast cancer patients and 23 healthy volunteers. We found that the IL-21 gene was overexpressed in breast cancer patients (P= 0.0005). The fold expression was 2.8 more than the control group, as shown in Table 6, and this result agreed with [10].

Also, we found that the gene expression of IL-21 is Significantly increased in the metastasis group than in the metastasis group, illustrated in Table 8. This result agrees with [7]; IL-21 and IL-21R promote migration and invasion of breast cancer cells by regulating PI3K/AKT signaling pathways.

CONCLUSIONS

Evidence exists that IL21 is implicated in various immunological processes. However, the participation of IL21 in the pathogenesis of solid tumors is not fully conclusive.

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