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# Estimation of *IL-21* Gene Expression Associated with Breast Cancer in Iraqi Patients

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**Abstract:** In this study, we intended to detect the expression of the Interleukin 21 (IL-21) gene in breast cancer patients and its influence on breast cancer molecular types (Luminal A, Luminal B, Her 2 enrich, and Triple-negative breast cancer (T.N.B.C) ) by collecting 5 ml of blood samples from a breast cancer patient who diagnosed by breast cancer and follows up in Al-Amal hospital and Al-Andalus private hospital and the number of patients was fifty pateints and the sample collection extended from 1 December 2021 to the 23 of February 2022 while twenty-Three healthy look volunteers were enrolled as a control group. The blood samples that were collected from patients and healthy volunteers were used to extract RNA and the molecular methods RT-PCR by using a specific primer for IL-21 gene.The result shows that there was overexpression in the sample of patients when compared with healthy volunteers and we found overexpression between Luminal A and T.N.B.C than Luminal B and Her2 enrich we found that T.N.B.C is a higher expression of IL-21 than other types of breast cancer.

**Keywords:** Breast cancer, interleukin 21, molecular types, Luminal A, Luminal B, Her 2 enrich, Triple-negative Breast cancer.

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## Introduction

Breast cancer is a heterogeneous disease (1). It is one of the leading causes of death from cancer worldwide (1.1 million cases are diagnosed annually) (2). Which conceder the second most common malignant tumor in females (3). Interleukin-21 (IL-21) is the latest addition to the common-gamma chain cytokine family. Primarily produced by natural killer T, T follicular helper (Tfh), and Th17 cells (4), IL-21 mediates a diverse range of regulatory functions on immune cells during allergy, cancer, and viral infection, IL-21 executes its anti-tumor function mainly through CD8+ T cells, NK cells, and regulatory T cells. IL-21 promotes the differentiation of Tfh and Th17 respectively (5). For B cells, IL-21 not only affects the survival of B cell (6).but also plays a role in class switching of the immunoglobulin (Ig) particularly promoting IgG1 and IgG3 (7). Direct regulation of BCL6 allows IL-21 to promote B cell proliferation and retention in germinal centers (GC) (8). The study also showed that IL21 expression by CD8+ T cells is required for antitumor immune response, IL-21 upregulated cell proliferation, motility, activation, and effector functions in $CD8^+$ T cells. The ability of IL-21 to induce $CD8^+$ T cells of a more memory phenotype. Specific $CD8^+$ induced by IL-21 have greater inhibition of tumor growth (9).

Breast cancer divided into surrogates of the genetically defined subgroups of breast cancers and the

subtype definitions were observed in table (1) as follows: luminal A (ER or PR+HER2-), luminal B (ER or PR+HER2+), HER2 overexpressing (ER-PR-HER2+), triple-negative (ER-PR-HER2-), basal-like (ER-PR-HER2-CK5+), non-classified (ER-PR-HER2-CK5-) and luminobasal (ER or PR+CK5+) (10).

**Table (1): Molecular subtype of breast cancer patents**

| Molecular subtype | | | | |
|---|---|---|---|---|
| **Immune profile** | **Luminal A** | **Luminal B** | **HER2/*neu*** | **Basal-like** |
| **ER, PR** | ER and/or PR+ | ER and/or PR+ | ER–, PR– | ER–, PR– |
| **HER2and others** | HER2– LowKi-67 (<14%) | HER2+or HER2– Ki-67 =14% | HER2+ | HER2– CK5/6and/or EGFR+ |

## Materials and methods

### Study design and Subjects

Fifty Iraqi women patients with breast cancer who attended Al-Amal hospital and Al-Andalus private hospital during the period extended from 1 December 2021 to the 23 of February 2022 with ages ranging from 30 – 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 represent different stages of the disease, and different age groups (30 – 67 years). All the cases were subjected to molecular study. Twenty-three apparently healthy volunteers with ages ranging from 26 - 63 years,wree also enrolled the study all twenty-three women were with no family history of breast cancer.

### Blood sample collection

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

### Molecular detection

RNA was extracted from the whole blood of breast cancer patients and healthy samples by using the TRIzolTM Reagent (Thermo fisher scientific USA) according to the manufacturer's protocol which include:

- Sample lysis: by mixing Each tube for 400 µl of blood and 600 µl of TRIzolTM Reagent,
- Purification: by add 0.2 mL of chloroform to each tube of lysate, After that incubating for 2–3 minutes at room temperature and centrifuging for 10 minutes at 12,000 rpm, the mixture was separated into three phases:organic phase, interphase, and a colorless upper aqueous phase.
- Preceptation: The aqueous phase containing the RNA was transferred to a new tube, then add 0.5 mL of isopropanol was to the aqueous phase, which was incubated for 10 minutes before being centrifuged for 10 minutes at 12,000 rpm.Total RNA was precipitated, resulting in a white gel-like pellet at the bottom of the tube.
- Washing: each tube, 0.5mL of 70 % ethanol was added and vortexed briefly before centrifuging for 5 minutes at 10000 rpm to remove any debris.After that, the pellet was aspirated and dried with air.
- RNA solubility: then put in Incubator at 55–60°C in a water bath for 10 minutes After that rehydrated the pellet in 20 µl of Nuclease Free Water.

- Quantus Fluorometer was used to detect the concentration of extracted RNA to detect the quality of samples for downstream applications. Include 1 µl of RNA to 199µl of diluted QuantyiFluor Dye was mixed. After 5 min incubation at room temperature in a dark place, RNA concentration values were determined.

## qRT-PCR

### Primer design and preparation

The sequence of (*IL-21*) gene obtained from NCBI gene bank (NG_031966.2) primer was designed with melting temperature ranging from 60-62 C$^{o}$ primer length was 22 nucleotides primers are supplied from Macrogen company and that shown in table (2).

**Table (2): Show the primers that are used in qRT-PCR of IL-21gene and TBP Housekeeping gene**

| Primer | Sequence (5`→3`) | Annealing Temp. (C$^{o}$) |
|---|---|---|
| IL-21 gene | CCAAGGTCAAGATCGCCACATG | 60 |
| | TGGAGCTGGCAGAAATTCAGGG | |
| Housekeeping gene | CAGTCTGCGAATGGTACTAA | |
| | TCAGTGGGGGTGAATTCAGTG | |

### One-step qRT-PCR protocol of *IL-21* gene

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 bioinstrumentation for gene level measurement. Several applications have already been implemented in the field of cancer research, and others are being validated, showing that this molecular biology tool can provide both researchers and clinicians with precious information concerning the behavior of tumors (11). In this experiment, the (QRT-PCR) protocol is done by adding the following reaction ingreadiants (master mix, RT mix, MgCl2, CXR, Nuclease free water, forword primer, Recers primer and RNA) according to manufactural instruction, and the reaction program of one step QRT-PCR is done according to manufactural instraction RT enzyme activation 37 C$^{o}$ for 15 min RT enzyme inactivation at 95 C$^{o}$ for 10 min , denaturation 95 C$^{o}$ for 10 sec , annealing 60 C$^{o}$ for 30 sec and extension at 72 C$^{o}$ for 30 sec.

### Statically analysis

The static analysis is done by Microsoft excel 2016 by 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 significant statically (12).

## Results and discussion

This study involved 60 cases of breast cancer patients and 23 healthy volunteers the present study showed 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 figure (1) this result is agreed with (13) who use some immune gene signature to high-risk breast cancer. In their report, they found that prognostic significance is limited to HR+/HER2− breast cancer. Proliferation-based gene signatures are strongly prognostic for ER+/HER2−

breast cancer, but less so for other subtypes of breast cancer There results show that IL-21 (Interleukin 21) and IL21R (interleukin 21 receptor) play a role in promoting migration and invasion of breast cancer (14). Another study shows that IL21R expression by CD8+ T cells is required for the antitumor immune response of anti-ErbB2 antibodies against HER2+ tumors; also, IL21 signaling via IL21R may increase trastuzumab efficacy (15).

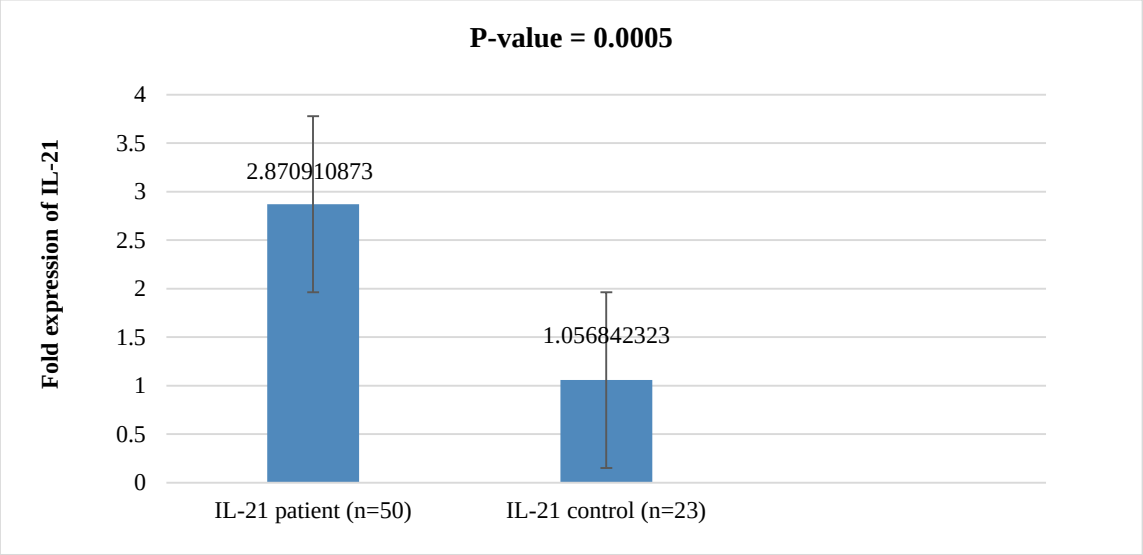


**Figure (1): Fold change (2^-ΔΔCt) of IL-21 expression of patents and control group.**

The fold expression of *IL-21* gene is significantly associated with patients categorized in Luminal A or T.N.B.C than the patents with Luminal B or Her 2 enrich and this result is matched with the result of (13) they found that patents with Her 2 neu positive they have decrease in the expression of IL-21, the down-regulation of IL-21 gene expression with patents with molecular type Her-2 neu is matched with the result of (15,16) they found that using IL-21 with the cancer treatment will be helpful to increase cancer healing.

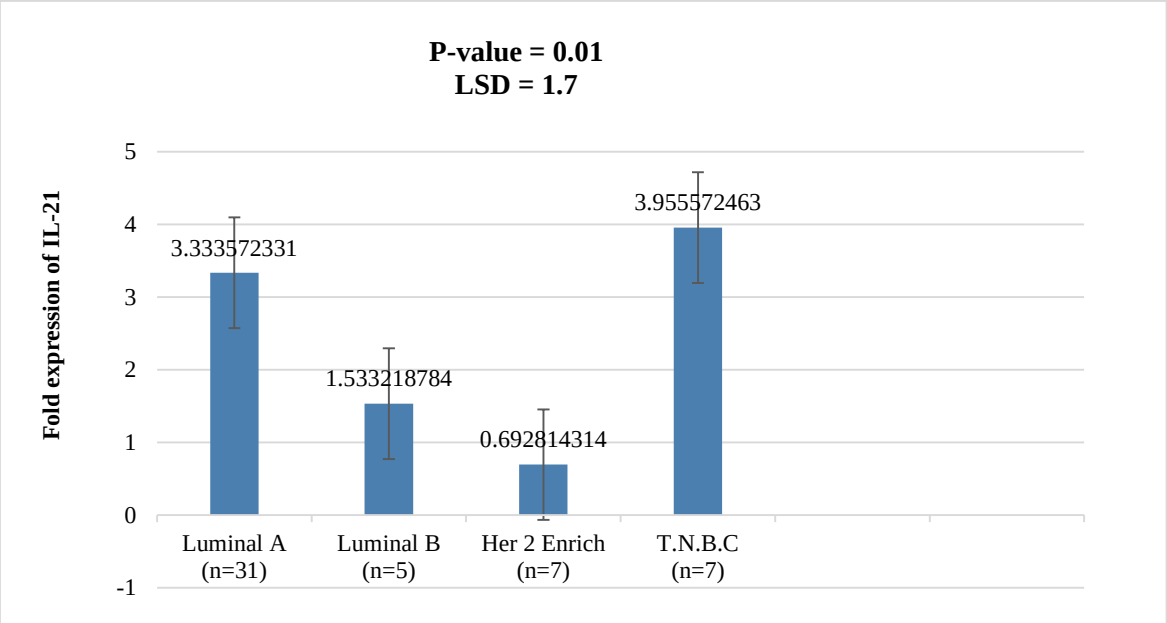


**Figure (2): Fold change (2^-ΔΔCt) of IL-21 expression according to molecular types of breast cancer.**

## Conclusion

Interleukin 21 is a prognostic cytokine that plays role in immune responses by regulating the function of T cells, B cells, natural killer (NK) cells, or interleukin signaling pathways IL-21 is overexpressed in patients with good prognostic type Luminal A and T.N.B.C poor prognostic type (13).

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