Iraqi Journal of Biotechnology, 2023, Vol. 22, No. 1, 140-147

The Role of Long Non Coding RNA *ANRIL* Gene Expression and Serum Interleukin-27 Level in Metastasis of Breast Cancer Patients

[1]Ali K. Ibrahim, [2]Mohammed I. Nader, [3]Sana A. Abbood

[1]AL-shaheed AL-Sadir General Hospital, Baghdad
[2]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad
[3] AL-Emamein AL-Kadhmain Medical City

Received: March 1, 2023/ Accepted: April 11, 2023/ Published: June 4, 2023

Abstract: Breast cancer is a heterogeneous disease and is the most common and prevalent form of malignancy diagnosed in women. The antisense non-coding RNA in the INK4 locus (*ANRIL*) belongs to long non-coding RNA family is overexpressed in several cancers including breast cancer. The study aims to understand the role of *ANRIL* in breast cancer this study investigated *ANRIL* expression in breast tumors using RT-qPCR. Additional assay for IL-27 were used in this study to evaluate the role of IL-27 in breast cancer metastasis. The study included 100 histopathologically confirmed with breast cancer. After blood collection, the serum was separated and divided into two aliquots, for total RNA extraction; cDNA synthesis and serum Interleukin-27 measurment. The *ANRIL* over expressed in patients with breast cancer (7.779940883) fold times compared to the healthy controls group (1.038805018). The fold change for the stages I, II, III and IV were (4.297018327, 5.553563985, 6.757504959 and 11.63189562) respectively. Twenty five cases with metastasis and fourty nine cases with lymph node involvement showed positive correlation with *ANRIL* expression. The levels of serum IL-27 were significantly increased in patients with breast cancer than in the control group (P=0.004). In addition, an elevation in serum IL-27 levels noticed in patients with metastasis (P=0.0001). Moreover, the serum levels of IL-27 was elevated in stage IV patients compared to stage I, II and III (P= 0.002, 0.0021 and 0.002 respectively). In conclusion these data indicate that *ANRIL* is highly expressed in malignant breast cells, and it may function in breast cancer prognosis, and the IL-27 may be a new prognostic biomarker of metastatic breast cancer.

Key words: Breast cancer, *ANRIL*, interleukin-27, gene expression.

Corresponding author: (Email: ali.khaleel1687@gmail.com).

Introduction

Breast cancer (BC) is multifactorial heterogeneous disease (1, 2), and it is the most prevalent type of cancer worldwide and the second leading cause of cancer mortality in women (3,4). According to the Statistics of cancer in the United States in 2020, BC accounts (30%) of cancers in women, with an estimated 276,480 new cases and more than 42,000 deaths in 2020 (5). Breast cancer is the primary cause of cancer among women and the leading cancer-related female mortality in Iraq (6). According to cancer board of Iraq (2020), the total number of breast cancers in both genders were (6255) per (100,000) of population (19.74%) and the first of top ten cancers in females with incidence (19.35%), (6132) per (100,000) of female population, while in male was (123) per (100,000) of population (0.39%), the mortality of BC accounting (6.75) per (100,000) female population and represent (25.54%) (7). Breast cancer diagnostic, prognostic, and predictive biomarkers are essential for the detection and appropriate management of the disease throughout therapy (8, 9).

Among the variety of biomarkers, the long non-coding RNSs (lncRNAs) and the interleukins (ILs) have an important role in human cancer development and progression, particularly in breast cancer (10, 11). Long non-codig RNAs are single-stranded RNAs that are longer than 200 nucleotides in length non-protein-coding transcripts molecules (12). LncRNAs participate in cellular processes such as differentiation, proliferation, apoptosis, migration, and epigenetic regulation (13). LncRNA *ANRIL* (antisense non-coding RNA in the INK4 locus) in the antisense direction is overexpressed in various cancers including breast cancer (14).

The heterodimer IL-27 was first described in 2002, secreted by antigen-presenting cells such as dendritic cells, monocytes, and macrophages, also by endothelial cells, the IL-27 consists of two subunits the α−subunit IL-27p28 and the β-subunit Epstein–Barr-induced gene 3 (EBI3) (15). IL-27 has tumor-promoting activities in many cancers, the elevation in the serum levels of IL-27 was reported in gastro-esophageal cancer, and it is highly expressed in invasive cutaneous melanoma, and breast cancer patients (16).

Materials and methods

Sample collection

One hundred Iraqi female participate in this study, 75 of them was pathologically confirmed as breast cancer patients aged between 30-75 years, and 25 were healthy control. The patients were grouped according to their stages to stage I 12 patients, II 13 patients, III 25 patients, and IV 25 patients, 49 Patients were positive for lymph node metastasis and 26 were negative. After blood samples collection the samples were centrifuged at (3000) rpm for 5 minutes to obtain serum (300) µL directly placed in Trizol preservation for RNA extraction and maintained at (-70°C) until used for the molecular investigation and (1000) µl of the serum stored at (-70°C) till examination of interleukin levels by ELISA.

Measurement of serum (IL-27) concentration

The serum IL-27 levels in the samples were measured using Sandwich-ELISA kit (Sunlong Biotech, China) by ELISA system (Dia Lab, Austria). The procedure was performed according to the manufacturer's instructions.

Molecular assays

RNA extraction

For total RNA extraction, Triquick Reagent (Solarbio, China) is used according to manufacturer instructions and protocol. For phase separation chloroform was used with a volume of 200 µl of and the aqueous was transferred into a new tube, then for the RNA precipitation 0.5 mL of isopropanol was added, the pellet was washed with 0.5 mL of 70% ethanol, the supernatant was discarded and the pellet was kept only, 50 µL of RNase-free water was added and the RNA kept in -70°C until RT-PCR reaction. Using Quantus Fluorometer (Promega, USA) the concentration of RNA in samples was quantified.

Reverse transcription for complementary DNA (cDNA) synthesis

AddScript Reverse Transcriptase kit (addbio, Korea) was used for the complementary DNA (cDNA) synthesis, in the 0.2 ml PCR tube the template RNA and cDNA Primer were added to each tube containing reverse transcription master mix, and gently mixed. The thermal cycle setting for reverse transcription was 25°C for 10 minute 1 cycle, 50°C for 60 minutes, and 80°C for 5 minutes 40 cycles. The

integrity of cDNA was detected by measuring its concentration using Quantus Fluorometer (Promega, USA) for downstream applications.

Quantitative real-time polymerase chain reaction (RT-q PCR)

Quantitative Real-Time Polymerase Chain Reaction (RT-q PCR) was performed using SYBR Green PCR Kit (AddBio, Korea) according to instructions of the manufacturer. The total volume of the reaction was 20 µL, containing 10 µL of SYBR Green Master Mix, 1 µL of specific primers 17 forward and reverse (table 1), 4 µL of cDNA 4 µL of RNase-free water, Real-Time PCR system (Bioer LineGene, China) was used to perform the reaction. The conditions of the cycling were set as follow: 95˚C for 1 min 1 cycle and 45 cycles of 95˚C for 20 seconds and 60˚C for 30 seconds. *ANRIL* expression levels were presented in terms of fold change normalized by *GAPDH* using the formula $2^{-\Delta\Delta CT}$.

Table (1): LncRNA *ANRIL* and *GAPDH* primers

Primer Name		Sequence 5´ -3´
ANRIL	**Forward**	CTCTCATCTGATCTCCGTCCT
	Reverse	TCACATCCAAGACAGCAAGT
GAPDH	**Forward**	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG

Statistical analysis

The normality distribution test of data was performed by Kolmogorov-Smirnov and Shapiro-Wilk. Categorical data were expressed as numbers and percentages, whereas the nonparametric variables were expressed as the median and interquartile range (IQR). Kruskal-Wallis H and Mann-Whitney *U* test was employed to determine the significant differences among medians of study groups. Receiver operating curve (ROC) analysis was employed to calculate the area under the curve (AUC), 95% confidence interval (CI), cut-off value, sensitivity and specificity; the Youden index was employed to optimize the cut-off value. The differences were considered significant when the P value ≤ 0.05. The statistical analysis was performed using IBM SPSS Statistics 26.0.

Results and discussion

Molecular analyses of LncRNA *ANRIL* expression level

The RT-q PCR results for LncRNA *ANRIL* were analyzed by the relative quantification of gene expression levels (folding changes) based on the (Ct) values. The breast cancer group showed a high level of *ANRIL* expression (7.779940883) compared to the healthy controls group (1.038805018) as shown in (figure 1, Table 2), stage I, II, III and IV (4.297018327, 5.553563985, 6.757504959 and 11.63189562) respectively as shown in (Figure 2, Table 2). In correlation to lymph node status the results showed and upregulation in *ANRIL* expression in patients with lymph node metastasis with mean fold change (9.594527628) compared to patient with no lymph node involvement that showed lower expression (4.360142787) (Figure 3, Table 2).

Many studies have reported upregulation in *ANRIL* gene in different types of cancer; Lui, *et al.* (18) reported overexpression of *ANRIL* in gallbladder cancer. Lin *et al.* (19) and Nie *et al.* (20) aslo reported an increase in the expression of *ANRIL* in the cancer of lung. The over expression of *ANRIL* was reported by Alkhathami, *et al.* in

the advanced stages of breast cancer patients than in the early stage (21). Mehta-Mujoo, *et al.* (22) reported the upregulation of *ANRIL* expression in TNBC than other breast cancer types. Numerous other studies suggest that the high expression of *ANRIL* could be employed as a predictive factor for lymph node metastasis in human cancers; Wang *et al.* (23) suggested that there was significant correlation of *ANRIL* overexpression with lymph node metastases in BC patients. Sun *et al.* (24) found high expression of *ANRIL* in lymphatic metastasis of colon cancer. Some evidence suggests that high *ANRIL* expression can promote various biological behaviors, such as proliferation, migration, invasion, and the epithelial-mesenchymal transformation, *ANRIL* can also suppress apoptosis to a certain degree (19, 24).

The lymphangiogenesis is driven by *ANRIL* via the upregulation of *LYVE1*, *VEFG-C*, and *VEGFR-3*, by the binding of *VEGF-C* to *VEGFR-3* or hyaluronan binding to *LYVE1* lymphangiogenesis is started (24). The polycomb group proteins are gathered directly by *ANRIL* to *INK4a* and *INK4b* loci, reducing *p16INK4a*, *p14ARF*, and *p15INK4b* expression. The *p16INK4a* and *p15INK4b* are cyclin-dependent kinase 4 and *p14ARF* inhibitors (23). These mechanisms suggest that the high expression of *ANRIL* is associated to breast cancer progression and metastases.

Table (2): The fold change of *ANRIL* expression.

Healthy control		Breast cancer	
1.038805018		7.779940883	
Stage I	**Stage II**	**Stage III**	**Stage IV**
4.297018327	5.553563985	6.757504959	11.63189562
Lymph node Negative		**Lymph node Positive**	
4.360142787		9.594527628	

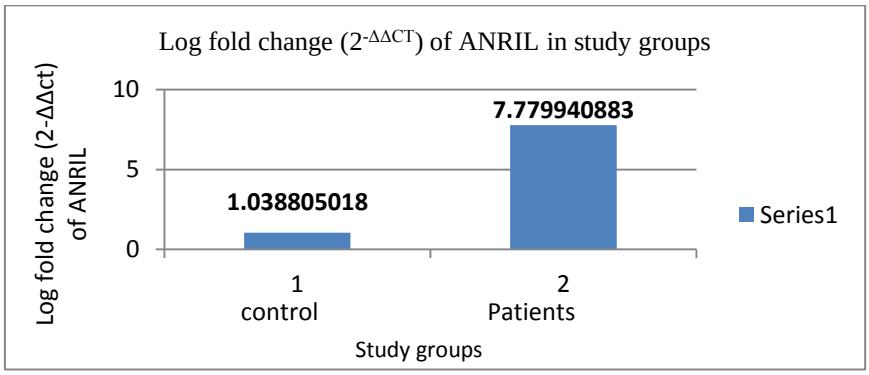

Figure (1): Log fold change ($2^{-\Delta\Delta CT}$) of *ANRIL* in study groups.

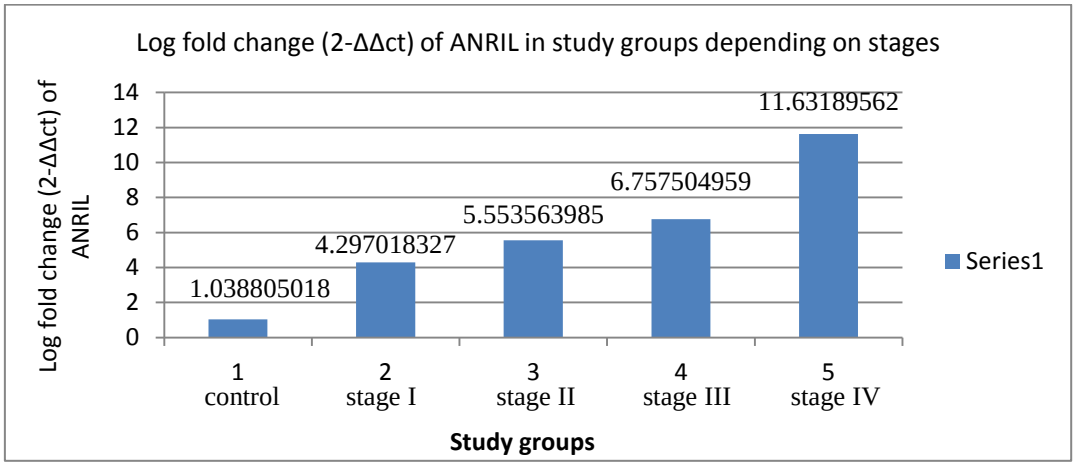

Figure (2): Log fold change ($2^{-\Delta\Delta CT}$) of *ANRIL* depending on stages.

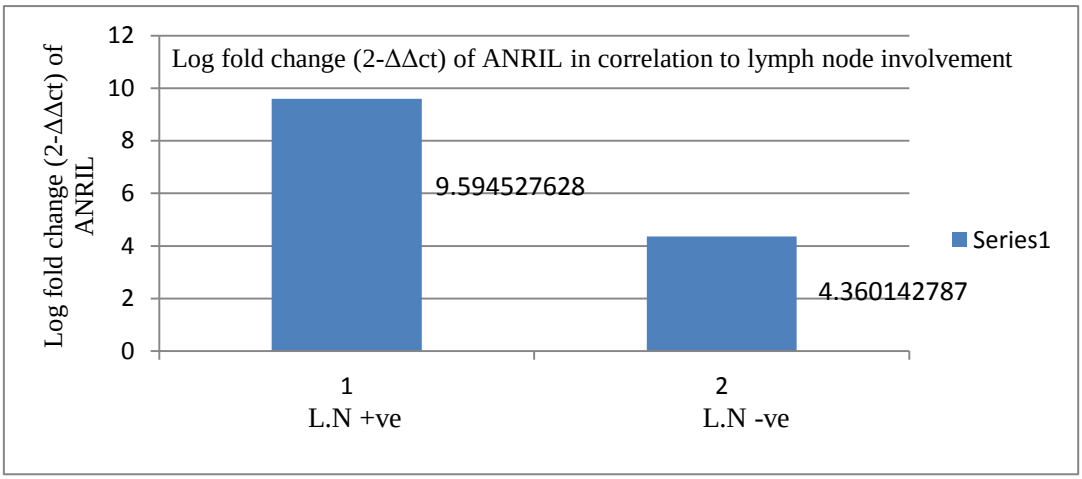

Figure (3): Log fold change (2-ΔΔct) of *ANRIL* in correlation to lymph node involvement

Serological assays for serum IL-27 concentration

The median (IQR) of the serum IL-27 level of the breast cancer group 17.8814 (10.169) pg/ml which was higher than the control group 14.4915 (11.0169) pg/ml, the median of the serum IL-27 level of stage IV 20.4237 (11.016) pg/ml was higher than that of stage III, II and I, which were 14.9153 (7.838), 17.8814 (8.474) pg/ml and 16.6102 (8.262) pg/ml respectively.

The results show a significant increase in the IL-27 level between breast cancer group 17.8814 (10.169) pg/ml compared to the control group 14.4915 (11.0169) (P=0.004) (table 3). The IL-27 level of stage IV 20.4237 (11.016) pg/ml was significantly higher than control group (P=0.0001), also higher than stage I (p=0.02), stage II (p=0.021), and stage III (p=0.02) (Table 4).

Table (3): IL-27 level in patients group *vs* control group.

Descriptive Statistics	Serum concentration of IL-27 (pg/ml)	
	BC patients	HC group
Median (IQR)	14.4915 (11.0169)	17.8814 (10.169)
P value	0.004	

Table (4): IL-27 levels in study groups via Mann-Whitney.

Descriptive Statistics	Serum concentration of IL-27 (pg/ml) in stage IV *vs* HC		
Median (IQR)	20.4237 (11.016)		
P value	0.0001		
Stages	**IV *vs* I**	**IV *vs* II**	**IV *vs* III**
P value	0.02	0. 0.021	0.02

Many studies reported high levels of serum IL-27 in different types of cancer; Babadi *et al.* observed an increase in serum IL-27 level in patients with lung cancer in comparison with the control group (25). Yuan *et al.* (26) reported significant increase in serum levels of IL-27 in Hepatocellular Carcinoma. Many studies reported high levels of serum IL-27 in breast cancer patients than those of the healthy group (16, 27). A possible mechanism leads to elevated levels of IL-27 is may be due to the cytotoxicity of dendritic cells against living human tumor cells is prevented by apoptotic tumor cells by inducing IL-27, thus stimulating a regulatory T cell population (27). And there are a few studies that address the effect of IL-27 on tumorogenesis, IL-27 has also been linked to tumor progression (28, 29). These results suggest that IL-27 may be a biomarker in breast cancer progression.

The diagnostics performance of IL-27 marker in the studied groups

Receiver Operator of Characteristics (ROC) curves analysis of serum IL-27 was found with an fair sensitivity of (71.6%) and poor specificity of (60.9%) at the area under the curve (AUC= 0.700) and Cut off value (14.9153) pg/ml (Table 5 and Figure 1). These results with the good AUC make the IL-27 reliable biomarker to be used in the assessment of breast cancer progression.

Table (5): ROC curve analysis of miRNA-21 to distinguish between BC and HC

Parameter	Cut off value	sensitivity	Specify	Area Under the curve (AUC)
IL-27	14.9153pg/ml	71.6%	60.9%	0.700

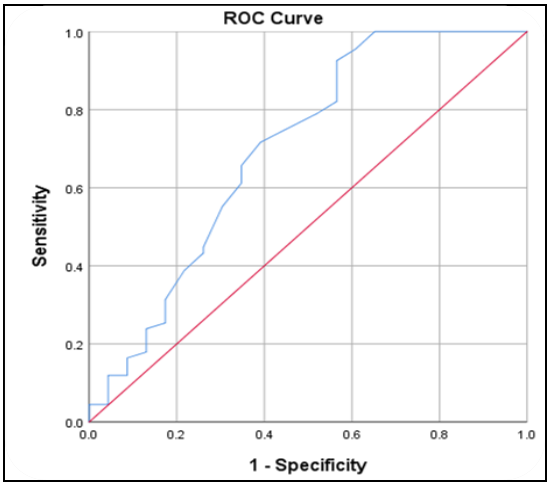

Figure (1): ROC curve analysis of IL-23 levels in patients *versus* controls.

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