Arch Iran Med. March 2021;24(3):209-217

doi 10.34172/aim.2021.32

http://www.aimjournal.ir

ARCHIVES OF IRANIAN MEDICINE

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Original Article

Expression of *SCUBE2* and *BCL2* Predicts Favorable Response in *ERα* Positive Breast Cancer

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Abstract

Background: The study aimed at evaluating steroid biomarker genes (*ERα, PGR, ERβ*) and determining the expression level of estrogen-regulated genes (*SCUB2* and *BCL2*) and growth factors receptors (HER2 and IGFR1) in cancer tissue samples obtained from Iranian patients with breast cancer. Moreover, relationships with clinicopathologic aspects of tumor and response to treatment were studied.

Methods: The current study was conducted on 246 breast tissue samples. The expression levels of these genes and their relationships with clinicopathologic aspects and treatment response were evaluated.

Results: Based on immunohistochemistry (IHC) results, 12% of the ER negative patients expressed ERα. Comparing the effects of ERα and coexpression of *BCL2* and *SCUBE2* on the survival of the patients demonstrated remarkably poorer survival in ERα positive, SCUBE2, and BCL2 negative groups in comparison with other patients, which was statistically significant in the log-rank analysis ($P = 0.01$). Evaluation of the effects of coexpression of *HER2* and *IGFR1* on patients' survival demonstrated a worse survival rate in patients with positive expression of both receptors, which was insignificant.

Conclusion: Many studies suggest that PGR alone is not enough for the functional evaluation of ERα. Evaluation of the progesterone receptor expression as well as other genes such as *BLC2*, *SCUBE2*, and *IGFR1*, seems necessary to evaluate functionality.

Keywords: *BLC2*, *ER*, *IGFR1*, Multigene model, *SCUBE2*

Cite this article as: Esmaeili R, Mohammadi S, Jafarbeik-Iravani N, Yadegari F, Olfatbakhsh A, Mazaheri M, et al. Expression of *SCUBE2* and *BCL2* predicts favorable response in *ERα* positive breast cancer. Arch Iran Med. 2021;24(3):209–217. doi: 10.34172/aim.2021.32.

Received: May 31, 2020, Accepted: October 19, 2020, ePublished: March 1, 2021

Introduction

Today, cancer studies are focused on finding diagnostic, predictive, and prognostic biomarkers. These biomarkers are commonly assessed in tumor tissues by categorizing patients into different subgroups leading to the selection of effective therapeutic methods. Since breast cancer (BC) is a heterogeneous disease, finding such biomarkers is of great importance to personalize the treatment.

Most of the clinically approved biomarkers are assessed using immunohistochemistry (IHC). Nevertheless, some recently developed cancer genetic panels such as Oncotype DX employ quantitative real-time polymerase chain reaction (qRT-PCR) to assess biomarkers.[1] Several studies reported some practical biomarkers to predict the efficacy of different therapeutic approaches for BC such as hormone therapy, targeted therapy, and chemotherapy.[2-5] However, none of them are approved for clinical practice yet.

In about 70% of patients with BC, estrogen receptor alpha (*ERα*) is expressed in the early tumor, which indicates that tumor cells growth is hormone-dependent and such patients can benefit from endocrine treatment; moreover, tamoxifen is the standard choice in most of the patients with ERα$^+$ patients. Nevertheless, the prognosis of ERα$^+$ cases is different, and some of them develop resistance against treatment after the therapeutic course, while the mechanism of this resistance is not entirely understood.[6] Expression of progesterone receptor (*PGR*) is another factor; in other words, *PGR*$^+$ indicates that the *ER* signaling pathway is functional in such patients. *PGR*$^+$ in tissue samples is a positive predictive factor and indicates the functionality of the *ERα*$^+$ pathway and the patient may gain maximum advantage from blocking the pathway.[7,8] Most of the studies recommend that *PGR* alone is not enough for the functionality of *ERα*.[9] According to a variety of responses to therapeutic methods in *ERα*$^+$/*PGR*$^+$ patients and sometimes lack of response to treatment in the early stages, the expression of signal peptides, CUB

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domain, and EGF such as domains containing 2 (*SCUB2*) and B-cell leukemia/lymphoma 2 (*BCL2)* as well as *PGR* is of great importance.[10,11] These genes are suggested as estrogen-regulated genes.[12,13] Some studies suggest measuring these markers to predict hormone therapy.

In addition to hormone therapy, the expression of biomarkers plays a significant role in responding to other therapeutic agents such as chemotherapy and targeted cancer therapy. For example, it was observed that in addition to *HER2*, blocking the expression of *ER* and insulin-like growth factor receptor-1 (*IGFR1*) influences response to treatment with herceptin.[14] It was also observed that lower expression of *IGFR1* after chemotherapy is associated with better response to treatment in patients undergoing ACT (adriamycin, cyclophosphamide, and taxotere) chemotherapy.[15] It is noteworthy that higher expression of *IGFR1* exacerbates response to chemotherapy and higher activity of *IGFR1* protein induces resistance to radiotherapy and chemotherapy.[16]

In recent years, the expression of *ERβ*, the second identified estrogen receptor, is evaluated in different studies and the results are rather controversial.[17-19] Tumors with higher levels of *ERβ* expression have a lower risk of an event such as recurrence or metastasis compared with tumors with lower expression levels in patients undergoing chemotherapy.[20]

The current study aimed at evaluating steroid biomarker genes (*ERα, PGR, ERβ*) and determining the expression level of estrogen-regulated genes (*SCUB2* and *BCL2*) and growth factor receptors (*HER2* and *IGFR1*) in cancer tissue samples obtained from Iranian patients with BC using real-time PCR. Also, their relationships with clinicopathologic aspects of tumor and response to treatment were evaluated.

Materials and Methods

Tissue and Sample

The current study was conducted on 246 breast tissue samples including 123 tumors and 123 normal adjacent tissues. Sample size was calculated using an online web tool with 95% confidence level, 80% margin of error and 10% population proportion.[21,22] The sample size was calculated at 62 for each group. Due to the possibility of sample attrition, 123 samples were considered in each group. Tissue samples along with clinicopathologic data were obtained from the Breast Cancer Research Center Biobank (BCRC-BB), Iran. According to the protocols followed by BCRC-BB, immediately after excisional biopsy or surgery, sample tissues were snap-frozen in liquid nitrogen and stored at -70°C.[23]

Primers and TaqMan probes were designed by Gene Runner software version 3.0.5 for *ERα*, *PGR*, *ERβ*, *SCUBE2*, *BCL2*, *HER2,* and *IGFR1*. The list of primers and probes are available upon request. *ACTB* and *TFRC* were used as housekeeping genes.[24]

RNA Extraction and cDNA Synthesis and Gene Expression Assay

RNA extraction was performed using 820 mg of the breast tumor and normal adjacent tissue by Rnx-Plus (Cinagen, Iran) as previously explained.[24] The quality and quantity of the extracted RNA were measured by gel electrophoresis and spectrophotometry, respectively. The cDNA synthesis was performed using the cDNA synthesis Kit (Qiagen, Germany) according to the manufacturer's protocol. Real-Time PCR was conducted using SYBR Green Master mix (Takara, Japan) and ABI 7500 version 2.0.6. Normal adjacent tissue was used as control.

Data Analysis

Gene expression was analyzed using the 2-ΔΔCT method. Gene expression amounts >2 were considered as upregulation. Data were analyzed using SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). The student *t* test was used to compare ΔCT between tumor and normal adjacent tissue. The frequency of genes expression was presented in total samples and also in ER- and ER+ positive patients separately (ER protein expression production based on IHC results). Age and follow-up time were presented as median/IQR and median/range respectively. Clinicopathologic data were presented as categorical data with frequencies and percentages. Shapiro Wilk's and Levene tests and probability plots were used to evaluate the normal distribution of the variables and the homogeneity of variances, respectively. One-way analysis of variance (ANOVA) was employed to conduct the comparisons among the three groups.

The chi-square or the Fisher exact tests were used to determine the significance of differences between up- or downregulated gene expression and clinicopathologic variables. The correlation between gene expressions and clinicopathologic data was evaluated by following the Shapiro-Wilk's method for normality tests and Pearson's for correlation tests using the Pearson correlation test. The Kaplan-Meier analysis with log-rank tests was performed to calculate the cumulative survival proportion for disease free survival (DFS) based on the gene expression level. A Cox proportional hazards model was applied to investigate the univariate hazard ratio. Date of surgery was assumed as time zero. For multivariate analysis, variables with $P < 0.2$ in univariate analysis were included. $P < 0.05$ was considered to specify a statistically significant result.

Results

Patients

Demographic and clinicopathological data of the patients including age at diagnosis, IHC results of estrogen, progesterone and HER2 receptors, stage, grade, lymph nodes, and tumor size are summarized in Table 1. The number of triple negative subtypes in comparison with other subtypes is also included. The median age of the

Table 1. Demographic and Clinicopathologic Data of the Patients (n = 125)

Age (median/IQR) (y)	**48 (14.75)**
IQR (Q3, Q1)	(54.75,40)
Follow-up (median/range) (mon)	48.5 (1–65)
ER (9 missing), No. (%)	
Negative	44 (37.9)
Positive	72 (62.1)
PR (10 missing), No. (%)	
Negative	53 (46.1)
Positive	62 (53.9)
HER2/neu (11 missing), No. (%)	
Negative	81 (58.0)
Positive	33 (23.7)
Grade (13 missing), No. (%)	
G1	12 (10.6)
G2	72 (63.7)
G3	29 (25.7)
Patient status (7 missing), No. (%)	
Healthy survival	92 (78.0)
With events	26 (22.0)
Stage(22 missing), No. (%)	
I	9 (8.7)
II	47 (45.2)
III	35 (33.7)
IV	13 (12.5)
Tumor size (13 missing), No. (%)	
< 2 cm	30 (29.8)
2–5 cm	65 (58.0)
>5	17 (15.2)
Lymph node (14 missing), No. (%)	
No	32 (28.8)
1–3	33 (29.7)
4–9	31 (27.9)
>9	15 (13.5)
Subtype, No. (%)	
Triple negative	22 (19.4)
Other	91 (80.6)

IQR, Interquartile range.

patients was 48 years (range 29-87), and median follow-up was 48.5 months (range 1-65).

Gene Expression

The frequencies of upregulated, downregulated, and no changes in gene expressions are summarized in Table 2. It was observed that *ERα* and *ERβ* were also expressed in ER^- patients (based on IHC results). Twelve percent of ER^- patients expressed *ERα*. Also, the expression of this gene was absent in 5% of ER^+ patients, but *ERβ* expression was almost the same in ER^+ and ER^- groups. This pattern was observed for *PGR*, *SCUBE2,* and *BCL2* expression as estrogen related genes. Some ER^+ patients lacked the expression of these genes, while some ER^- patients expressed the abovementioned genes. There was a significant difference between tumor and matched normal tissue samples in the expression of *BCL2*, *ERβ*, *HER2*, *PGR*, and *SCUBE2*. *P* values are 0.0001, 0.0001, 0.005, 0.0001, and 0.0001, respectively.

The ANOVA analysis showed that patients with larger tumor sizes expressed higher *HER2* ($P = 0.028$) and lower *ERβ* levels ($P = 0.029$). Moreover, patients above 50 years expressed higher *HER2* ($P = 0.05$) and lower *ERβ* ($P = 0.047$). It was also observed that tumors with lower levels of *ERα* expression had higher grades ($P = 0.032$) (data not shown).

Correlation of Gene Expressions and Clinicopathologic Data

As shown in Table 3, a significant negative correlation was observed between *ERβ* expression and tumor stage ($P = 0.006$). Moreover, upregulation of *HER2* was correlated with higher stage and tumor size ($P = 0.028$, $P = 0.041$).

The correlations between different genes are shown in Table 4. There was a significant correlation between *ERα* and *PGR* ($P < 0.001$) and *SCUBE2* ($P < 0.001$), and *SCUBE2* and *IGFR1* ($P < 0.001$). There were also significant negative correlations between *ERα* and *IGFR1* ($P < 0.001$), *ERβ* and *HER2* ($P < 0.001$), and PGR and IGFR1 ($P < 0.001$). The two latter correlations were strong. Other correlations were not significant. Chi-square analysis showed that upregulation of *IGFR1* was associated with triple negative subtype ($P = 0.01$) (data not shown). Analysis of *BCl2*, *SCUBE2*, and *ERβ* showed no significant association with the type of tumor.

Survival Analysis and Prognostic Significance of Gene Expressions

Association between the selected gene expressions and patient survival was evaluated using the Kaplan-Meier analysis with a log-rank test for DFS. The median follow-up duration was 48.5 months (95% CI: 1–65 months). According to this analysis, gene expression status had no significant impact on survival of patients. Prognostic values of all gene expressions were investigated in the univariate and multivariate analyses of DFS. Although upregulation of *ERα*, *HER2,* and *PGR* showed negative effects on survival and upregulation of *BCL2*, *IGFR1*, *SCUBE2,* and *ERβ* had positive effects on survival, the results were only significant for *ERα*. Multivariate analysis was not significant for the selected biomarkers (Table 5). Comparison of the effects of *ERα* and coexpression of *BCL2* and *SCUBE2* on patients' survival demonstrated a remarkably poorer survival rate in $ERα^+$, $SCUBE2^-$, and $BCL2^-$ groups in comparison with other patients, which was statistically significant in the log-rank analysis ($P = 0.01$). Comparison of the effects of *HER2* and *IGFR1* coexpression on patients' survival demonstrated a worse survival rate in patients with positive expression for both

Table 2. Frequency of Gene Expression in ER- and ER+ Patients

	N*	Percent	N (ER- Patients)	Percent	N (ER+ Patients)	Percent
ERα						
Down	52	41.6	24	54.5	25	35.2
No change	16	12.8	9	20.5	5	7
Up	57	45.6	11	22.7	41	57.7
ERβ						
Down	33	26.4	12	27.3	18	25.4
No change	8	6.4	2	27.34.5	5	7
Up	84	67.2	30	68.2	48	67.6
PGR						
Down	66	52.8	26	59.1	35	49.3
No change	17	13.6	3	6.8	13	18.3
Up	42	33.6	15	34.1	23	32.4
SCUBE2						
Down	86	68.8	26	59.1	52	73.2
No change	14	11.2	7	15.9	7	9.9
Up	25	20	11	25	12	16.9
HER2						
Down	94	75.2	32	72.7	53	74.6
No change	9	7.2	1	2.3	7	9.9
Up	22	17.6	11	25	11	15.5
IGFR						
Down	60	48	18	40.9	37	52.1
No change	14	11.2	177	15.9	6	8.5
Up	51	40.8	19	43.2	28	39.4
BCL2						
Down	60	48	25	56.8	31	43.7
No change	26	20.8	9	20.5	14	19.7
Up	39	31.2	10	22.7	26	36.6

*N, N (ER- Patients) + N (ER+ Patients) + missing.

Table 3. Pearson Correlation Coefficient for Different Gene Expressions and Clinicopathologic Data

Gene	Stage	Grade	LN	T Size	Status	Age at Diagnosis
ERa	-0.048	-0.086	0.047	-0.078	0.143	-0.04
ERb	-0.271**	0.048	-0.183	-0.168	-0.065	-0.15
BCLJH2	-0.041	0.026	-0.029	0.018	-0.10	0.06
HER2	0.220*	-0.023	0.177	0.193*	0.037	0.21
PGR	0.084	-0.046	-0.066	0.092	0.064	-0.16*
SUBE2	-0.066	0.121	0.001	-0.065	-0.067	-0.063
IGFR1	-0.084	0.049	0.48	-0.083	-0.023	0.05

* *P* value ≤ 0.05, ** *P* value ≤ 0.01.

Table 4. The Correlations between Different Gene Expressions

	ERaCAT	ERbCAT	PGRCAT	SCUBE2CAT	HER2CAT	IGFRCAT
ERaCAT	1.000					
ERbCAT	0.032	1.000				
PGRCAT	0.315**	0.164	1.000			
SCUBE2CAT	-0.469**	0.128	-0.201*	1.000		
HER2CAT	-0.099	-0.882**	-0.174	-0.089	1.000	
IGFRCAT	-0.388**	-0.127	-0.902**	0.311**	0.145	1.000

* *P* value ≤ 0.05, ** *P* value ≤ 0.01.

receptors, but the result was not significant (P = 0.1) (Figure 1). Coexpression of *ERβ* and *IGFR1* and other combinations of gene expression was not significant.

Discussion

In the current study, the expression of *ERα, PGR, ERβ, SCUBE2, BCL2, HER2,* and *IGFR1* genes was investigated using real-time PCR. The findings demonstrated the significant effect of coexpression of *Bcl2* and *SCUBE2* on the survival of patients with *ERα* overexpression. To the best of the authors' knowledge, the effect of coexpression of these two genes was reported only in the recurrence score calculation in Oncotype DX along with 18 other genes, which calculated the risk of distant metastasis in

Table 5. Univariate and Multivariate Analysis of Prognostic Impact of Both Clinicopathologic Parameters on Disease Free Survival

Overall Survival	Univariate			Multivariate		
	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI	*P* Value
ERα						
No and Down	1			1		
Up	2.90	0.91–9.2	0.07	2.27	0.685–7.583	0.180
ERβ						
No and Down	1			1		
Up	0.54	0.22–2.68	0.2	0.58	0.203–1.686	0.321
Her2						
No and down	1					
Up	1.40	0.44–4.5	0.54			
PGR						
No and down	1					
Up	1.80	0.65–5.3	0.23			
SCUBE2						
No and down	1					
Up	0.89	0.20–4.01	0.88			
IGFR1						
No and down	1					
Up	0.69	0.23–2.08	0.52			
BCL2						
No and down	1					
Up	0.74	0.23–2.3	0.62			
ER IHC						
Negative	1					
Positive	0.70	0.25–2.2	0.6			
PR IHC						
Negative	1					
Positive	0.80	0.27–2.4	0.7			
HER2 IHC						
Negative	1					
Positive	1.20	0.38–4.1	0.69			
Stage						
I and II	1			1		
II and IV	3.20	0.82–12.4	0.09	1.05	0.418–2.658	0.911
Grade						
I and II	1					
III	1.70	0.54–5.8	0.3			
Tumor Size						
I and II	1			1		
III and IV	2.60	0.8–8.9	0.10	2.49	0.692–8.996	0.162
LN						
No and I-III	1			1		
IV-IX and >IX	2.87	0.86–9.56	0.08	1.46	0.413–5.195	0.554
LN						
Negative	1					
Positive	5.10	0.66–40.1	0.1			
ER IHC						
Negative	1					
Positive	1.30	0.44–3.97	0.6			
PR IHC						
Negative	1					
Positive	1.20	0.40–3.6	0.7			
HER2 IHC						
Negative	1					
Positive	0.80	0.24–2.56	0.7			

CI, confidence interval

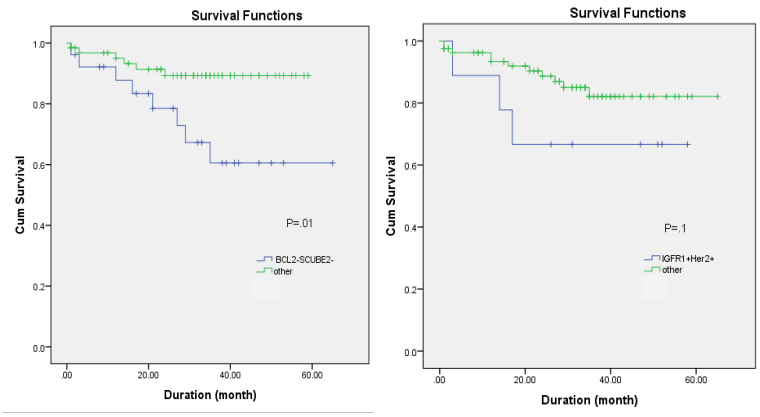

Figure 1. The Kaplan-Meier Plot. The Kaplan Meier plot of patient survival stratified by coexpression of BCl2- SCUBE2 (right) and IGFR1-HER2 (left) in ERα+patients; vertical marks show censored patients. The censoring was due to missing data of the patients' follow-up (0.2 in BCl2- SCUBE2 and 0.06 in IGFR1-HER2). Censoring means the total survival duration for that patient cannot be accurately determined. Most of the time, it occurs when participants are either excluded or refuse to participate in the study.

patients with stage I and II.[1] Moreover, a negative effect of coexpression of *IGFR1* and *HER2* on patients' survival was observed. Other studies show that blocking *IGFR1* is helpful for response to herceptin.[14] Moreover, patients with decreased expression of *IGFR1* had fewer events in chemotherapy.[15] Furthermore, increased expression of this protein had a negative impact on resistance to chemo- and radiotherapy.[16,25] The current study did not investigate the effects of gene expression on different treatment regimens due to unavailability of this data. The results of the current study showed that upregulation of *ERα*, *HER2,* and *PGR* had negative impacts on survival. Also, the effect of *BCL2*, *IGFR1*, *SCUBE2*, and *ERβ* upregulation on survival was investigated. However, the results were positively significant for *ERα*.

The prognostic effect of *ERα*, *PGR*, and *HER2* gene expression was in accordance with the effect of ER, PR, and HER2 protein by IHC. But there was no correlation between IHC and real-time PCR results of these biomarkers. It should be noted that recent studies have shown different correlations between RT-qPCR and IHC for ER. So, this discordance could be attributed to several factors, as follows. There is a lot of variation in IHC methodology. These wide variations consist of the cold ischemia time which is related to tissue processing, scoring and annotation system and using differences antibody clones,[26-28] all of which are different between labs. Moreover, the lack of a proper cutoff set for IHC as a semi-quantitative technique with different scoring methodology can cause this disparity.[29] The IHC technique targets ER protein. However, RT-qPCR evaluates ER gene expression at the RNA level. Therefore, post-translational modification of the ER gene may be responsible for this discrepancy. Tumor dissection is necessary for RT-qPCR assay. Sometimes, incorrect tumor dissection can lead to diverse results, indicating the inclusion of some parts of normal tissue which contaminates the sample.[29] It should be mentioned that in the current study, tumor dissection was performed only on the tumor part. In the Oncotype Dx test which evaluates the ten-year risk of recurrence in breast cancer, the real-time PCR assay is used for detecting the level of hormone receptors as well as HER2 which shows mRNA expression would be a good choice for diagnostic tests.

The results of the correlation between gene expression and clinicopathologic data of the patients were in concordance or disconcordance with some studies, which will be discussed.

According to previous studies, IGFR1 protein binds to the high-affinity insulin-like growth factor. This receptor has tyrosine activity and is overexpressed in many malignant tissues and acts as an anti-apoptosis factor, which increases the survival rate of cancer cells. In the current study, higher *IGF1R* expression was a favorable prognostic factor; its expression also had a positive correlation with *SCUBE2* expression and a negative correlation with *ERα* and *ERβ* expressions. The results of similar studies are in agreement with those of the current study, although some are inconsistent. LaTonia et al indicated a correlation between increased *IGF1R* expression and higher grades of the tumor, shorter DFS, and poor prognosis.[30] Some other studies also reported a correlation between the expression and activity of *IGFR1* with disease progression, increased resistance to radiotherapy, and poor prognosis.[31,32] Higher *IGFR1* expression in tumors >2 cm, and grade II or III was also reported by Browne et al.[33] A study by Yerushalmi et al showed that the expression of *IGFR1* was associated with lower tumor grades, ER expression, and lack of *HER2* expression. Expression of *IGF1R* was associated with good prognostic factors such as older age at diagnosis, lower grades, negative HER2, and higher levels of *P27*.[34] In contrast, a study on 60 patients with BC showed a significant relationship between the increased *IGF1R* expression and higher tumor grades. They suggested that

the overexpression of *IGFR1* was associated with invasive behavior of tumor cells, but indicated no relationship between *IGF1R* and stage of the disease or lymph node metastasis. It was also revealed that *IGF1R* expression might cause angiogenesis by vascular endothelial growth factor, and consequently, metastasis in BC cases.[35] Nevertheless, some similar studies indicated no association between the expression of *IGF1R* and clinicopathological features of the tumor; in a study on 210 paraffin-embedded early BC tumors, similar to the current study, no relationship was observed between IGF1R expression and clinicopathological features of the disease such as age at incidence, tumor size, lymph nodes status, and hormone receptors.[36] However, in a study by Al Sarakbi et al, a relationship was observed between the mRNA level of *IGF1R* and that of lymph nodes.[37] In a study on non-small cell lung cancer cases, increased IGFR1 expression was associated with larger tumor sizes.[38]

The SCUBE2 protein belongs to the SCUBE protein family and is a tumor-inhibitory factor. Expression of SCUBE2 protein has been observed in different tissue such as breast ducts epithelium.[39] Although the role of this protein is not perfectly identified in healthy cells, its expression is observed in early BC tumors, and better prognosis is reported in patients expressing *SCUBE2*, compared with the ones who do not express it.[11] The SCUBE2 protein inhibits tumor growth through the bone morphogenic pathway and beta-catenin signaling pathway.[39] In the current study, a negative correlation was observed between the expression of *ERα* and *PGR* with *SCUBE2* and also a negative correlation with the expression of *ERα* and *PGR* with *IGFR1*. Also, a significant correlation was observed between the increased expression of this gene and better prognosis in patients with BC. A study by Skrzypczak et al showed that the expression of this gene was reduced in cases with endometrial cancer of higher grades. It is noteworthy that the expression of this gene showed a positive correlation with the expression of *PGR* and *ER*. Another study also indicated lower recurrence and better survival rates in patients with colorectal cancer and higher expression levels of these genes; on the other hand, decreased expression of *SCUBE2* was associated with progression and prognosis in such patients.[40]

ERβ belongs to the estrogen receptors family and it is present in nucleus, cytoplasm, and mitochondria. By binding a ligand to ERβ, it forms homo- and heterodimers, which activate transcription from specific sequences of DNA. Some isoforms of this receptor inhibit the activity of other members of the estrogen receptors family.[41] In the current study, *ERβ* gene showed lower expression levels in tumors with a greater size. Also, patients aged above 50 years had lower *ERβ* and higher *HER2* expression levels. In a study by Sapino et al on BC using IHC and real-time PCR techniques, the mean age of ERβ+ patients was lower than those with ERβ-, which is in concordance with the findings of the current study.[42] Miyoshi et al reported that the *ERβ* expression was associated with tumors smaller than 2 cm and higher grades.[43] The first result is in concordance with the findings of the current study. Another study showed that smaller size tumors and higher overall survival (OS) were associated with lack of *ERβ* expression.[44] In a study on ovarian cancer, *ERβ* expression was associated with metastasis to lymph nodes. By evaluating 508 tumor samples, no significant association was observed between *ERβ* expression and clinicopathological features.[45]

In conclusion, many studies today suggest that PGR alone is not enough for the functional evaluation of Erα,[21] based on the variety of responses to treatment in $ER\alpha^{+}/PgR^{+}$ patients and even lack of response to different treatments in the early stages. Evaluation of the progesterone receptor expression as well as other genes such as *BLC2* and *SCUBE2*, *IGFR1* (using the multigene model), seems necessary to evaluate the functionality of ERα.[15,22]

Authors' Contribution

RE designed the project, was responsible for data analysis and writing the manuscript. SM, NJ, and MR performed the experiments. AK and AO were in charge with clinical data. KM conceived and designed the project and critically reviewed data analysis and the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

None declared.

Ethical Statement

The project was approved by the Ethics Committee of the Breast Cancer Research Center (BCRC). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Funding

This study was funded by ACECR (Academic Center for Education, Culture and Research) and grant number is 1828-11.

Acknowledgements

The authors would like to acknowledge the ACECR for financial support.

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