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Evaluation of *PALB2* Gene Expression in Breast Cancer

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Abstract

Breast cancer is the most prevalent malignancy and the second in mortality rate cancers among all cancers in women globally. In Iran it adds up to 16 percent of all cancers and is the most prevalent cancer in Iranian women. *PALB2*, identified as a partner and localizer of *BRCA2* and proposed to have some roles in DNA damage response, has recently been identified as a breast cancer marker gene. Considering the importance of the breast cancer in Iranian women population, in the present study we aim to make clear whether the expression level of the *PALB2* could be accounted as a biomarker in timely diagnosis and also a deterministic factor in prognosis of breast cancer. Accordingly, the real-time PCR has been used to evaluate *PALB2* expression in blood samples acquired from 40 cancer patients and 10 normal women. Having been investigated in this Iranian women population with breast cancer, the *PALB2* gene expression has shown a borderline significant increase, as compared to normal cases (*P* value = 0.0499). Furthermore, in this study, the correlation between *PALB2* gene expression and several risk factors in breast cancer including age, menstrual state, family history of breast cancer as well as family history of other cancers has also been studied.

Keywords: Breast cancer; *PALB2*; Gene expression analysis; Real-time PCR.

Introduction

The most prevalent malignancy among women with approximately 1.67×10^6 new cases diagnosed in 2012 worldwide is the breast cancer [1]. It is characterized by unchecked proliferation of aberrant cells in mammary milk-secreting glands or milk-carrying ducts to the nipple [2]. Breast cancer is the first cause of mortality in the developing world and the second in the developed countries [3]. Although the prevalence rate is lower in Iranian population, with one case from each 10 to 15, the age of onset is, at least, a decade lower compared to the developed countries [4].

As a heterogeneous disease, breast cancer develops from a mutual interaction of hereditary and environmental risk factors leading to progressive

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accumulation of genetic and epigenetic alterations within affected cells [5]. The genetic abundance, life style, and environmental factors are influencing on breast cancer risk, among all, the genetic holds the greatest importance. The genes implicated in breast cancer are divided into medium to high, and low penetrance genes, reflecting the relative risk in carrying mutations. The most recognized group of genes with medium to high penetrance are *BRCA1*, *BRCA2*, *P53*, *PTEN*, *STK11*, and *CDH1* that elevate the cancer risk up to four fold [6]. There are other genes also linked to increased risk of breast cancer. These genes that tend to be low penetrant and less implicated in breast cancer, often have a role in DNA repair pathways, maintenance of genome integrity, and cell cycle checkpoints. The 2 to 4 fold higher breast cancer risk is concomitant with the mutations in *RAD50*, *PALB2*, *NBS1*, *CHEK2*, *BRIP1* and *ATM* genes [7]. As the partner and localizer of *BRCA2*, a cancer susceptibility gene, *PALB2* has been recently recognized as a breast cancer gene that may have a role in DNA damage response [8]. *PALB2* located on chromosome 16p12.2 with an approximate length of 38kb including 13 exons and 12 introns, and encodes a protein with a role in BRCA2 related pathway [9]. Loss-of-function mutations in *PALB2* gene occurred in 1 to 2 percent of women with breast cancer in several populations that shows a lower potential, as compared to *BRCA1* and *BRCA2*, to increase the hereditary breast cancer risk. It has been suggested that *PALB2* has a prominent role in the process of complex formation between *BRCA1* and *BRCA2* which guides their proper functioning in the DNA damage signaling pathway. This has attracted a great deal of attention to the *PALB2* role in DNA damage response pathway and, hence, the consequence of its, mutations in cancer [8].

Considering the importance of breast cancer issue as one of the main causes of mortality, globally, and researches conducted as to the role of the *PALB2* gene in breast cancer, the present study deals with the *PALB2* expression level in female breast cancer patients in the Iranian population.

Materials and Methods

The present study conducted on 50 venous blood samples of subjects including 40 female breast cancer patients and 10 female normal control cases. All the cases were residing in Farda laboratory (Tehran, Iran). All cases were between the ages of 20 and 78 years.

Normal samples included in the present study, were taken from individuals in the age between 20 to 75 years. 40% of patients (16 individuals) have family history of breast cancer, while the percentage for those who have family history of other cancers is 47.5% (19 individuals). 47.5% of patients (19 individuals) were over 50 years old and 52.5% (21 individuals) were under 50 years old (Table 1).

RNA extraction: 500µl of RNX-PLUS solution has been added to each 100µl blood sample. The mixtures have been left at room temperature for 5 minutes and then 200µl chloroform added to each. After 25 minutes centrifugation in 12000 rpm at the 4℃, the upper, and colorless phase separated and added to equal volume cold isopropanol and left for 2 hours at −20℃. Subsequently, the mixture centrifuged in 13000 rpm at 4℃ for 10 minutes and the liquid phase removed. The samples went for another round of centrifugation in 13000 rpm for 10 minutes right after addition of cold 70% ethanol solution. The liquid phase has been removed and the former step repeated one more time before letting the sample dry at room temperature. 30-50µl DEPC-treated water has been added to samples and stored at −20℃.

cDNA synthesis: To a 10µl extracted RNA solution, 1µl of random hexamer primers alongside 1µl Oligo-dt and 1µl dNTP has been added and incubated for 5 minutes at 65℃ in thermocycler (Convergent technologies, Germany). The mixture stored at 4℃ for 1 minute immediately after the former step and then after addition of 4.5µl DEPC, 0.5 µl M-MuLV reverse transcriptase enzyme (CinnaGen, Iran), and 2µl M-MuLV buffer, the mixture stored for an hour at 42℃ in the thermocycler.

Primer design: Sequences of *PALB2* (NM_024675.3) and *GAPDH* genes from NCBI reference sequence database have been used to design the relevant primers by Primer Express™ software. To verify the designed primers, NCBI and BLAST (Basic Local Alignment Search Tool) algorithms have been used. The primer sequences has been shown in Table 2.

Real-time PCR: 10µl of master mix containing 0.2µl

Table 1. Characteristics of breast cancer patients and normal individuals analyzed in this study

Total cases	Normal cases	Patient cases	Patients with B.C family history	Patients with family history of other cancers	Patients upper 50 years	Patients under 50 years	Menopausal patients	Non-menopause patients
50	10	40	16	19	19	21	25	15

Table 2. The sequences of primer used in this study

Primer	Sequence	T_m ℃	Amplicon size (bp)
PALB2 (F)	TGGGTGTGATGCTGTACTGTCTT	61.83	141bp
PALB2 (R)	GGGCAGTACACTGACCGAGA	61.25	
GAPDH (F)	ATGGAGAAGGCTGGGGCT	60.29	124bp
GAPDH (R)	ATCTTGAGGCTGTTGTCATACTTCTC	60.85	

Table 3. Real-time PCR programs

Gene	Cycle 1	Cycle 2	
		Step 1	Step 2
PALB2	5 min (95℃)/1 repeat	40 repeats/15s (95℃)	40 repeats/1min (60℃)
GAPDH	10 min (95℃)/1 repeat	45 repeats/15s (95℃)	45 repeats/1min (59℃)

forward primer, 0.2µl reverse primer, and 5µl cDNA and 4.6µl PCR-grade water has been put into the real-time PCR (ABI-7500, USA). To normalize the expression a house keeping gene, GAPDH, has been used. The time and temperature programs have been set for both *PALB2* and *GAPDH* according to Table 3.

Statistical analysis: In the present study the expression level of target gene, *PALB2*, has been compared in patients relative to normal cases. Graphpad prism 7 has been used to analyze the results statistically.

Results

Evaluation of the *PALB2* gene expression using real-time PCR with SYBR green color indicates a modest increase in *PALB2* expression level in patients as compared to normal cases. To calculate ΔΔct, the sample ct has been calculated after replication phase and subsequently turned into relative quantification (RQ). The expression level of *PALB2* was 1.8 fold higher in patients relative to normal cases (*P* value = 0.0499) (Figure 1).

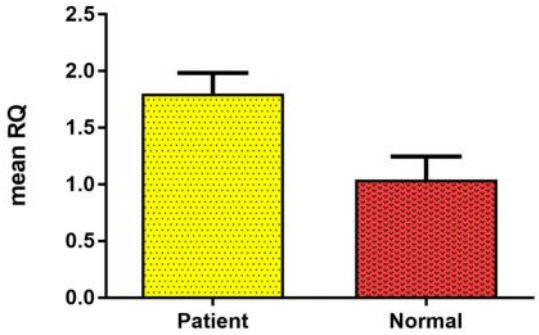

Figure 1. The *PALB2* gene expression graph of patients and normal cases. *PALB2* have shown more expression in patients than normal cases (*P* value = 0.0499).

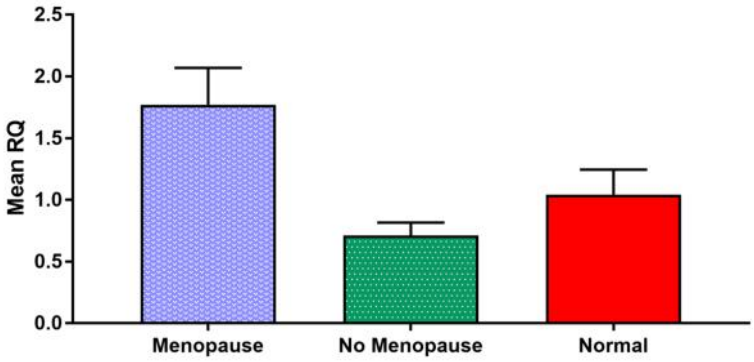

Figure 2. The *PALB2* gene expression graph based on menopause in patients. The *PALB2* gene expression in menopausal patients was significantly more than non-menopausal patients (*P* value = 0.0054).

Furthermore, the correlation between *PALB2* gene expression and several risk factors in breast cancer including; age, menstrual state, family history of breast cancer as well as family history of other cancers has been studied (Table 4). Among all these risk factors, menopause was the only factor with significant association to *PALB2* gene expression such that in average there was a 1.77 fold increase in expression in menopause patients and 0.71 fold decrease in non-menopause patients relative to normal cases (*P* value = 0.0054) (Figure 2).

In addition, the association of *PALB2* expression with breast cancer clinical symptoms has been assessed. Patients with each of the mentioned symptoms A-D, has been divided into 3 groups: 1. group A with clinical symptom A (a lump in the breast or under the armpit without pain), 2. group B/D with clinical symptoms B (nipple indentation) and D (asymmetric breasts) and 3. group C/E with clinical symptoms C (shooting pain in the nipple area) and E (nipple bleeding). The results have indicated the *PALB2* expression level has an increase of 2.06 and 1.42 fold in groups A and B/D, respectively. In contrast, the C/E group has showed a 0.88 decrease in expression of the *PALB2* gene as compared to normal cases. Regarding to these results, *PALB2* gene expression was proximately associated with breast cancer clinical symptoms (*P* value = 0.0491) (Figure 3).

Evaluation of *PALB2* gene expression with age in both patients and normal samples, have indicated that no significant association between age and breast cancer (*P* value = 0.8490) (Figure 4).

PALB2 gene expression level in patient with family history of breast cancer, and those patients lacking such family history was increased compared to control (1.45, and 1.51 times, respectively). Although there is a modest expression increase in both groups compared to normal people, but there is not significant association between *PALB2* gene expression and family history (*P* value = 0.1702) (Figure 5).

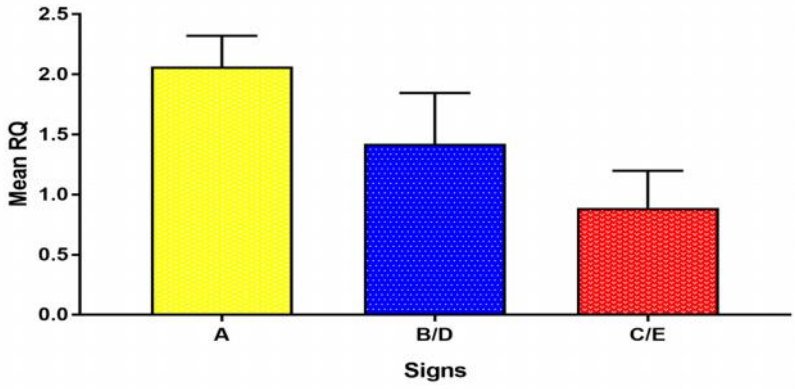

Figure 3. The *PALB2* gene expression based on clinical signs of breast cancer
(A) A lump in the breast or under the armpit without pain. (B) Nipple indentation. (C) Shooting pain in the nipple area. (D) Asymmetric breasts. (E) Nipple bleeding (*P* value = 0.0491).

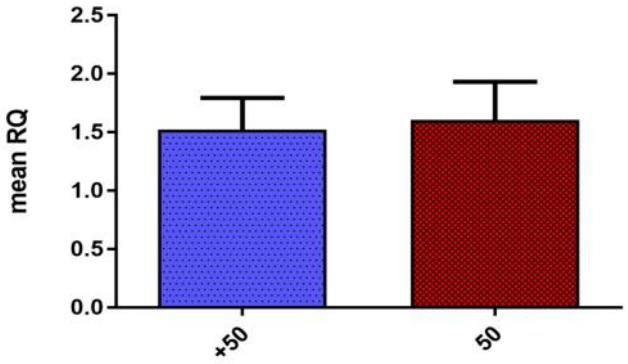

Figure 4. The *PALB2* gene expression graph is based on age in patient cases. No significant association was found between *PALB2* gene expression in patients over the age of 50 and under 50 years (*P* value = 0.8490).

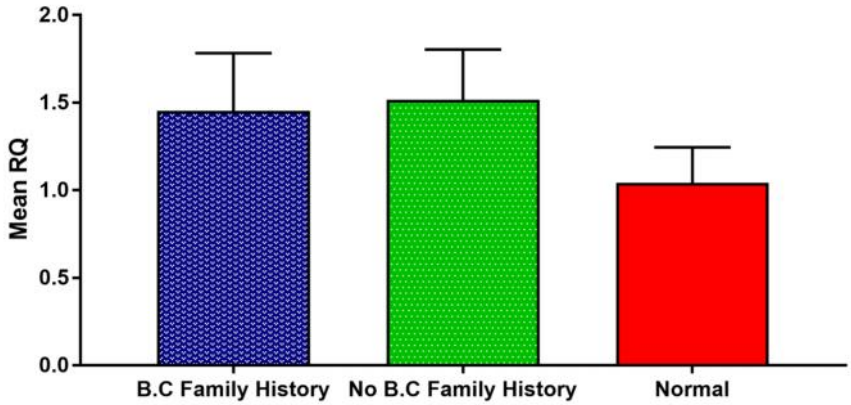

Figure 5. The *PALB2* gene expression graph based on breast cancer family history in patient cases. No significant association was found between *PALB2* gene expression and breast cancer family history (*P* value = 0.1702).

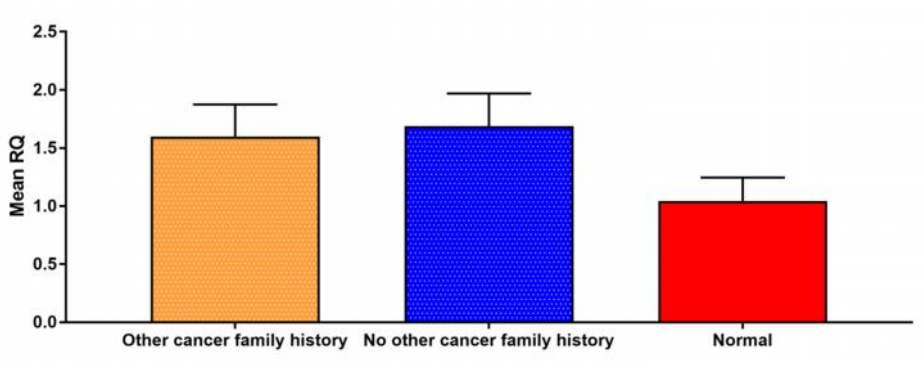

Figure 6. The *PALB2* gene expression graph based on other cancers family history in patient cases. *PALB2* gene expression was not found to associate with the other cancers family history in patient (*P* value = 0.8209).

Table 4. Associations between *PALB2* gene expression in patients and several risk factors in breast cancer

Factors	***P* value**
Gene expression in patients and normal cases	0.0499 *
Menopause in patients	0.0054 **
Signs	0.0491 *
Age	0.8490 ^{ns}
B.C family history	0.1702 ^{ns}
Other cancer family history	0.8209 ^{ns}

^{ns}: not statistically significant
*: statistically significant (*P* < .05)
**: not statistically significant (*P* < .01)

PALB2 gene expression in patients with or without family history of other cancers have showed 1.59 and 1.68 times increasing, respectively, compared to normal cases (*P* value = 0.8209) (Figure 6). Also *PALB2* expression level was not found to associate with the history of other cancers in the family of patients.

Discussion

Breast cancer is the second most prevalent cancer in women all around the world. Any progress toward early diagnosis of this malignant disease can be a valuable achievement in molecular medicine. PALB2, as a complex-forming proteins with the BRCA2, functions as an accessory protein in the BRCA1 and BRCA2 dependent DNA repair pathway as a defensive line against cancer development [10]. Today the *PALB2* is

considered as a well-established moderate to high penetrance breast cancer predisposition gene responsible for as much as 1-2.4% of diagnosed hereditary breast cancer[11]. It has been recognized as a potentially carcinogenic gene the assessment of which would be informative in genetic consultation of patients bearing a high risk of cancer development. Recent findings are suggestive that identification of *PALB2* variants might be illustrative in clinical prognosis and management of breast cancer patients [12]. In a study on expression level of *PALB2* gene in progressed breast cancer patients, Jingquan li and colleagues showed an elevated expression of *PALB2* in this group of patients [13]. In the present study, it has been also revealed that the *PALB2* expression level is higher in patients. Considering the direct interaction of PALB2 with key proteins in homologous recombination pathway, which has a major role in tumor suppression [14], it would be instructive to hypothesize this increase in *PALB2* expression is one probable mechanism in defense against cancer development. Additionally, the risk factor assessment associated with breast cancer has revealed that the *PALB2* expression has an increase in menopause patients which is in direct contrast to non-menopause patients with decreased amount of expression level as compared to normal cases. As it is a fact that women before menopause period are more susceptible to progressed types of breast cancer alongside the tumor suppressive role of *PALB2* gene, this decrease in *PALB2* expression in non-menopause patients would be more prominent and further research into its role as a tumor suppressor gene is in order.

One putative mechanism as to the *PALB2* expression level regulation is the epigenetic system with CpG island methylation as the most relevant one. In another study conducted by our group, in addition to *PALB2* expression level, it has been observed that there is a 92.5% promoter methylation and this hypermethylated state is accompanied with an increase in *PALB2* expression level in plasma samples of patients, a result which is in direct contrast to other studies on tissue samples that reported a decreased *PALB2* expression in a hypermethylated background (unpublished data). The reason behind this conflict would be the distinctive methylation patterns in plasma and tissue samples that could justify the opposing *PALB2* expression levels in a hypermethylated state. That this is the case demands further investigations as to the promoter methylation patterns in the *PALB2* gene in order to give a more clear insight regarding the role of CpG methylation in the pathogenesis of breast cancer.

As a consequence, considering the prevalence and high mortality rate of breast cancer as well as the proved role of *PALB2* gene in its pathogenesis, the present study dealt with the *PALB2* expression level in Iranian breast cancer women population with the aim of assessing the feasibility of targeting this gene for the purpose of diagnosis and prognosis determination. Accordingly, the results were illustrative the importance of *PALB2* expression level as a prognostic and diagnostic tool at the hands of the clinicians.

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