Research in Molecular Medicine

Comprehensive Analysis of ATP Synthase-binding Cassette Transporter Genes in Breast Cancer: Prognostic Value of *ABCD1*, *ABCA5*

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ABSTRACT

Background: ATP synthase-binding cassette (ABC) membrane transporter genes play a crucial role in mediating drug resistance and may serve as predictive biomarkers for treatment outcomes in breast cancer (BC). This study aimed to examine changes in the expression of these genes.

Materials and Methods: Transcriptome data from BC were extracted from the Cancer Genome Atlas (TCGA), normalized, and categorized into malignant and healthy samples. Immunohistochemistry data examined protein levels, and linear models were used to evaluate variations in gene expression. Kaplan-Meier curves and Cox regression analysis were used to investigate the correlations with survival. Data from PharmacoDB were utilized to examine the relationship between drug sensitivity and resistance. RNA extraction and cDNA synthesis were performed on samples from the Iranian Tumor Bank, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to evaluate the expression of *ABCA5* and *ABCD1*.

Results: Differential expression analysis identified eight ABC transporter genes with upregulated expression and 19 genes with downregulated expression in BC tissues compared to healthy samples. Specifically, high *ABCD1* expression was correlated with poor prognosis, whereas higher levels of *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2* were associated with improved patient outcomes. Protein expression analysis corroborated these findings. Co-expression network and pathway enrichment analyses revealed that *ABCD1* is involved in glycolysis, oxidative phosphorylation, and DNA repair pathways. The other candidate genes were linked to ABC transporter activity and fatty acid metabolism. Furthermore, ROC curve analysis demonstrated high sensitivity and specificity of the candidate genes in distinguishing malignant from normal tissues. Resistance to Docetaxel was linked to elevated expression of *ABCA5*, *ABCA8*, *ABCA10*, and *ABCB1*, which needs more confirmation. Our ex vivo studies revealed a notable difference in *ABCA5* and *ABCD1* levels between cancerous samples and healthy tissues.

Conclusion: This study highlights the potential of ABC transporter genes, particularly *ABCD1*, *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*, as novel prognostic biomarkers and contributors to drug resistance in BC. Their involvement in key oncogenic pathways underscores their significance in BC pathophysiology and warrants further investigation for therapeutic targeting.

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Introduction

The most frequent disease diagnosed in women worldwide, breast cancer (BC), is predicted to cause over 685,000 deaths and 2.3 million new cases in 2020 [1]. While survival rates have notably improved over the past two decades, the global incidence of the disease continues to rise [2]. Both hereditary and environmental elements play a role in the occurrence of BC. While some factors may be genetic, non-genetic contributors include radiation exposure, lifestyle choices (like alcohol use and obesity after menopause), age, reproductive risks (like early menarche and late menopause), external female hormones, and histological abnormalities, such as atypical hyperplasia [2, 3].

The adenosine triphosphate binding cassette (ABC) transporter superfamily members are found throughout all areas of life, facilitating substrate transport across cellular membranes [4]. The 44 membrane transporters encoded by the 48 ABC transporter genes in humans are divided into five families (A, B, C, D, and G) and exhibit a broad range of substrate specificities and functions [5]. The role of ABC transporters in facilitating drug efflux, which contributes to treatment resistance in BC, is widely recognized [6]. This process often involves essential transporters such as *ABCB1*, *ABCC1*, and *ABCG2* [7]. Reduced intracellular concentrations of chemotherapeutic drugs, such as vincristine and doxorubicin, may result from overexpression in cancer cells, worsening patient outcomes and treatment responses [8, 9]. Prognostic biomarkers may be derived from the expression levels of particular ABC transporters [10]. Elevated *ABCB1* levels are associated with unfavorable outcomes and treatment failures in patients with BC [11]. A multigene prognostic model incorporating ABC transporter expression can predict patient responses to neoadjuvant chemotherapy [12]. These findings highlight the significance of the ABC transporter family's expression and activity in BC development.

This research investigated the gene expression changes associated with ABC membrane transporters in BC. It explored their potential as diagnostic and prognostic biomarkers. Furthermore, the relationship between ABC membrane transporter expression and drug resistance was analyzed using in silico data, supplemented by ex vivo studies to validate the findings. This study provides insights into ABC family-related genes in BC, highlighting their association with prognosis and drug resistance.

Materials and Methods

Data sources

ABC family genes that may contribute to BC were found using the Cancer Genome Atlas (TCGA) data for BC. First, raw transcriptome data for BC (STAR-Counts) were retrieved [13]. Genes that showed no or nearly no expression in 70% of the samples (as indicated by a CPM of less than 10) were excluded from the data. The TTM method was employed to normalize the data, followed by a conversion of all values to a logarithmic scale based on 2 [14]. All analyses were conducted using the acquired expression matrix. This database included transcriptome data for 1109 cancer and 113 normal samples. Additionally, the clinical information of the samples was obtained. A review of the clinical data revealed that, of the 1109 cancerous samples associated with the TCGA database, 42 belonged to the HER2+ group, 456 to the luminal A group, 155 to the luminal B group, and 118 to the TNB group.

Differences in expression and examination of protein expression level of candidate genes

Initially, the samples were divided into two categories: Malignant and healthy, according to TCGA clinical data for BC. A linear model was utilized to assess the expression differences between cancerous and healthy samples, with the resulting p-values subsequently combined [15]. Different subgroups of BC were treated with the same technique. For example, the variation in expression of each gene, including candidate genes, was assessed in the HER2+ subgroup compared to the luminal A, luminal B, and TNBC subgroups. Additionally, the expression levels of proteins associated with candidate genes were analyzed using immunohistochemistry data from the Human Protein Atlas database [16].

Prognosis and survival rate

The normalized expression matrix and TCGA clinical data were used to study the link between ABC family gene expression and patient survival rate. Clinical data preprocessing included life status samples coded as 0, 1, and not available (NA) samples. Expression data for each gene in the normalized matrix were converted to Z-scores and integrated with clinical data. Finally, the Cox regression analysis assessed the relationship between patient prognosis and candidate gene expression. Based on median expression in cancerous samples, the samples were divided into high and low groups, with results confirmed using a Kaplan-Meier (K-M) curve.

Drug resistance and sensitivities

Using information from PharmacoDB, the association between drug resistance and sensitivity and the expression of putative genes was investigated. Two BC cell line databases, GRAY and UNH Breast, were utilized for this. The PharmacoGX software, version 3.14 was used to examine these two pharmacists [17]. Correlation coefficients greater than 0.4 and FDR <0.05 were used to select the relationships between medication resistance and sensitivity and the expression levels of candidate genes. Furthermore, the findings were considered in light of reviewer input, with more than 30 degrees of freedom.

Collection of samples

Table 1 summarizes the data from the 30 BC and 30 healthy samples used in this investigation. The Iranian Tumor Bank provided all the samples, and a pathologist verified that they were cancerous. All samples were obtained from colorectal cancer patients who visited Imam Khomeini Hospital in Tehran between 2017 and 2023. Tissue collection was performed during surgery, and none of the patients had received any drug treatment, chemotherapy, or radiotherapy prior to sampling. The Imam Khomeini Hospital review board examined and verified all bioethical concerns by Iran's Ministry of Health and Medical Education standards. Additionally, all potential patients provided written consent. Until they were used, all samples were kept in liquid nitrogen. The review team at Payam Noor University accepted every ethical case.

RNA extraction, cDNA synthesis, primer design, and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

As instructed by the manufacturer, the Trizol technique was used to extract each sample. The samples were repeatedly washed with PBS before extraction to remove tissue debris and necrotic cells. After treating the samples with DNase I to eliminate any DNA contamination, oligo (dT) and random hexamer primers were used for cDNA synthesis, following the manufacturer's instructions. The primers for *ABCA5* (F: 5'-TTTATATGGATTCAAGAGCTGGCTG-3' and R: 5'-TTAGTTGACTCCAGCTCCTTCCTTCC-3') and *ABCD1* (F: 5'-TGCAAAGGAAGGGCTCGCG-3' and R: 5'-ATTCTCTGCTTGCTTCGCCACC-3') were designed using the Primer-BLAST tool from the NCBI database [18]. In the primer design, the requirement for joining a primer at the exon-exon junction was considered to prevent DNA replication during qRT-PCR. Ultimately, the expression levels of the *ABCA5* and *ABCD1* genes in the examined samples were determined in triplicate using specifically designed primers and the SYBR Green technique. The expression level of the β-actin gene was used to standardize the data and provide an internal control.

Table 1. Clinical information of the participants

Subgroups	No. of Samples	Stage (No.)
HER2+	5	Stage I (1) Stage II (3) Stage III (1) Stage IV (0)
Luminal A	12	Stage I (2) Stage II (5) Stage III (3) Stage IV (2)
Luminal B	7	Stage I (0) Stage II (4) Stage III (1) Stage IV (2)
TNBC	6	Stage I (0) Stage II (4) Stage III (2) Stage IV (0)
Healthy	30	-

Statistics and software

All pre-processing of the TCGA raw data was conducted using the R programming language, software, version 4.3.2, employing the latest versions of the packages. The expression differences between groups were analyzed through the linear model method, applying a false discovery rate (FDR) threshold of <0.01. A one-way ANOVA was utilized to assess the significance of the qRT-PCR data. The log-rank method was used to evaluate the importance of survival-related tests. All graphs and figures were produced using GraphPad Prism software, version 8. AUC values exceeding 0.8 and P<0.01 indicated strong sensitivity and specificity, determined via the ROC curve.

Results

Significant expression changes in genes related to the ABC family in BC

The TCGA data examined the expression changes in every gene associated with the ABC family, as listed in the KEGG database [19]. Eight genes —*ABCA12*, *ABCA3*, *ABCB8*, *ABCB9*, *ABCC5*, *ABCD1*, *ABCG1*, and *TAP1*— exhibited higher expression among the 44 genes linked to ABC membrane transporters that met the logFC >0.5 and FDR <0.01 requirements (Figure 1A). However, 19 genes were identified as having significantly lower expression in malignant samples than in healthy ones. These genes included *ABCA1*, *ABCA10*, *ABCA13, ABCA4, ABCA5, ABCA6, ABCA8, ABCA9,* ABCB1, ABCB11, ABCB5, *ABCC2*, *ABCC6*, *ABCC9*, *ABCD2*, *ABCD4*, *ABCG2*, *ABCG8*, and *CFTR* (Figure 1A). Subsequently, the correlation between the identified genes and the patient mortality rate was examined. The findings demonstrated that among the elevated genes, increased *ABCD1* expression was associated with a worse prognosis for patients. Conversely, a favorable prognosis for patients was associated with higher expression of *ABCA10*, *ABCA5*, *ABCA8*, ABCB1, and *ABCD2* among the downregulated genes (Table 2). The K-M curve's findings also supported the previously stated points (Figure 1B). According to these findings, six ABC family genes —*ABCD1*, *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*— may be linked to the aggressiveness of BC and may be used as predictive biomarkers.

Expression level of proteins related to candidate genes and high sensitivity and specificity of the candidate genes

The Human Protein Atlas data from the previous phase were used to analyze the expression levels of proteins associated with the potential genes. The findings demonstrated that while the amount of protein associated with the *ABCA10*, *ABCA5*, *ABCA8*, and *ABCB1* genes was extremely low, the amount of ABCD1 protein was significantly elevated in cancer tissues (Figure 2A). These outcomes aligned with the expression level data from the previous step. Data on the protein expression levels of the *ABCD2* gene were not found. Additionally, the sensitivity and specificity of the expression of candidate genes in cancerous versus healthy samples were assessed. Compared to normal, the expression levels of *ABCD1*, *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2* in cancerous samples demonstrated good sensitivity and specificity, as illustrated in Figure 2B. These findings suggest that the candidate genes of the ABC family may be more significant in the pathophysiology of BC than those of other ABC family members.

Table 2. Results of cox regression analysis for clinical features and candidate genes

Genes	Univariate Cox			
	HR	95% CI for the HR		LogRank
		Lower	Upper	
ABCD1	1.42	1.13	1.78	0.002
ABCA10	0.65	0.48	0.88	0.005
ABCA5	0.77	0.6	0.98	0.03
ABCA8	0.72	0.54	0.94	0.01
ABCB1	0.68	0.52	0.9	0.002
ABCD2	0.69	0.51	0.93	0.01

HR: Hazard ratio.

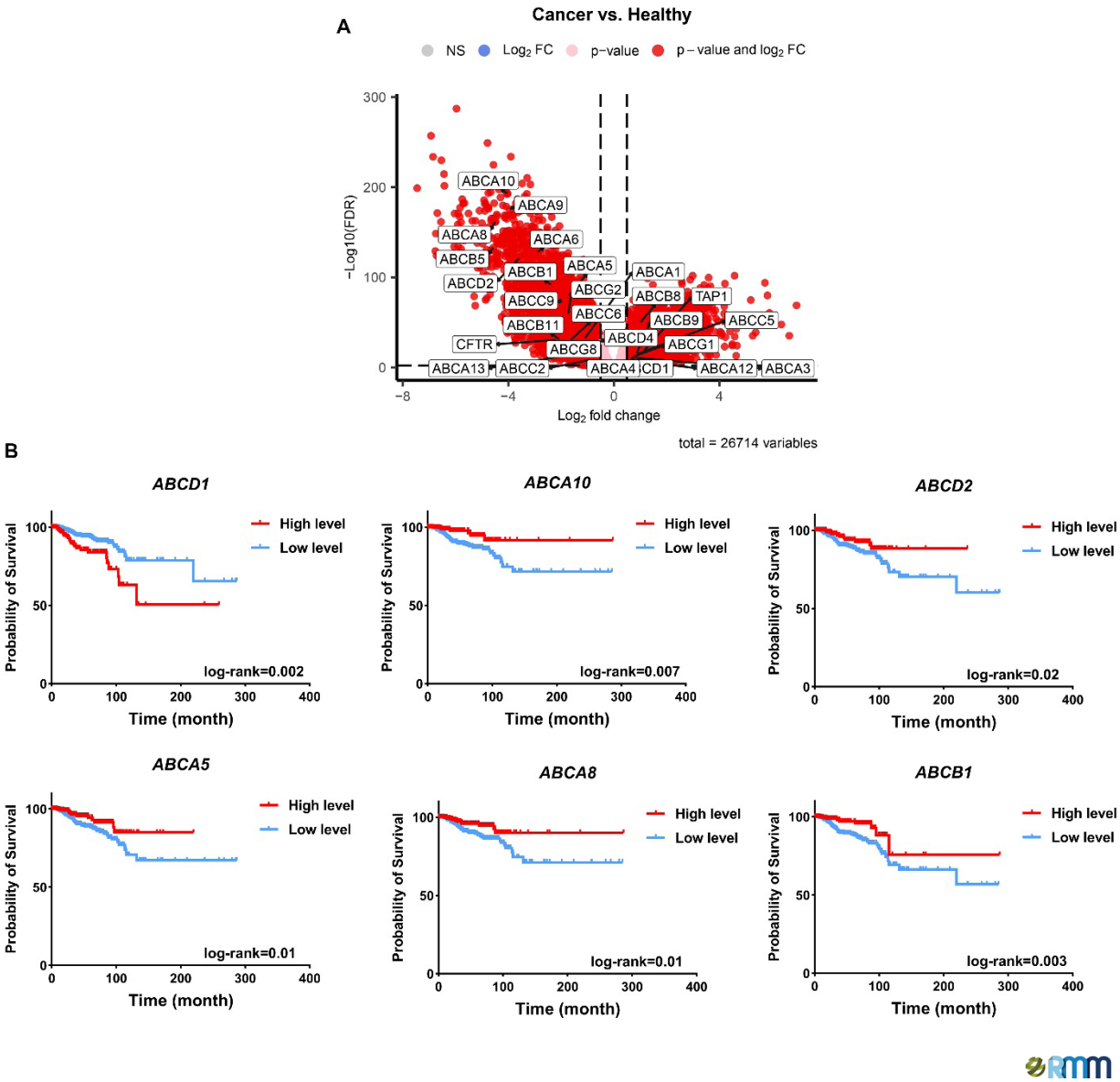

Figure 1. Altered expression of ABC family genes in BC and the relationship between their expression and patient survival rates

A) A volcano plot for differential expression of all genes related to the ABC transport family based on the TCGA data; the comparison was between cancer and normal samples; B) The expression of genes in the ABC transporter family was linked to the survival rate of BC patients

Association of the expression level of candidate genes with genes related to malignant pathways

A co-expression network was employed to determine the potential pathways that candidate genes from the ABC family may influence. The *ABCD1* co-expression network revealed that, generally, the expression level of this gene was correlated with that of 54 other genes (Figure 3A). According to the enrichment of these 54 genes, several play a significant role in processes, such as glycolysis, oxidative phosphorylation, and DNA repair (Figure 3C). However, 122 genes were found to be associated with the expression levels of *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*, and 122 genes were found to be involved in pathways, such as ABC transporters, fatty acid metabolism, and bile acid metabolism, according to the pathfinding results (Figures 3B and 3D). These findings suggest that the listed pathways may influence the candidate genes.

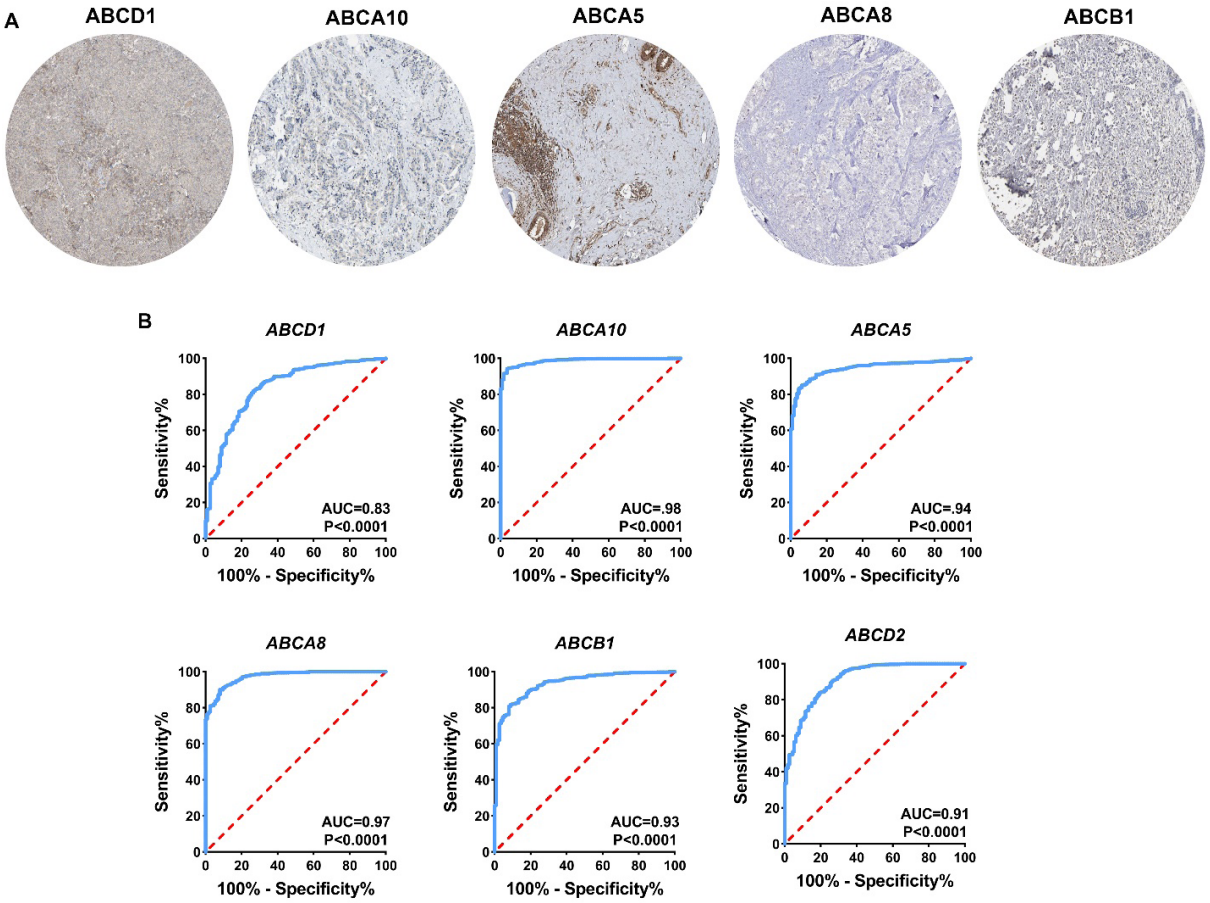

Figure 2. Expression changes of ABC family-related genes in BC as a diagnostic biomarker

A) The expression levels of proteins related to candidate genes are shown in the immunohistochemistry data from the Human Protein Atlas database; the findings demonstrated that while the quantity of proteins associated with the *ABCA10*, *ABCA5*, *ABCA8*, and *ABCB1* genes was extremely low, the quantity of proteins associated with the *ABCD1* gene was elevated in cancerous tissues; B) The sensitivity and specificity of candidate gene expression levels are demonstrated in differentiating cancerous samples from normal ones

The relationship between the expression of candidate genes and different subgroups of BC and changes in their expression in cancerous samples

Next, using TCGA data, the association between expression of candidate genes and various BC subgroups was examined. According to the *ABCD1* data, the HER2+ subgroup exhibited a higher gene expression level than the other subgroups (Figure 4A). Conversely, the luminal A subgroup exhibited higher expression levels of *ABCA8*, *ABCA10*, *ABCA5*, and *ABCB1* compared to different subgroups (Figures 4B, 4C, 4D and 4E). There was no evidence of *ABCD2* expression reliance among the various subtypes of BC. Furthermore, there was no correlation between stage features and any of the putative genes. To validate the results obtained, the qRT-PCR method was used to examine the expression levels of *ABCA5* and *ABCD1*, which are less evident in BC. According to qRT-PCR data, the expression level of *ABCA5* was considerably lower in cancerous samples than in healthy ones (Figure 4G). However, compared to healthy samples, cancerous samples showed higher expression of *ABCD1* (Figure 4F). These findings demonstrate that BC altered *ABCA5* and *ABCD1* expression, which may be associated with the disease. None of the *ABCA5* and *ABCD1* genes were related to distinct subgroups of BC, as indicated by the qRT-PCR results.

Association of increased expression of *ABCA5, ABCA8, ABCA10* and *ABCB1* with Docetaxel drug resistance

GRAY and UHNBreast data examined the association between candidate gene expression and medication resistance and susceptibility, as the ABC transporter family is crucial in these conditions. Interestingly, drug re-

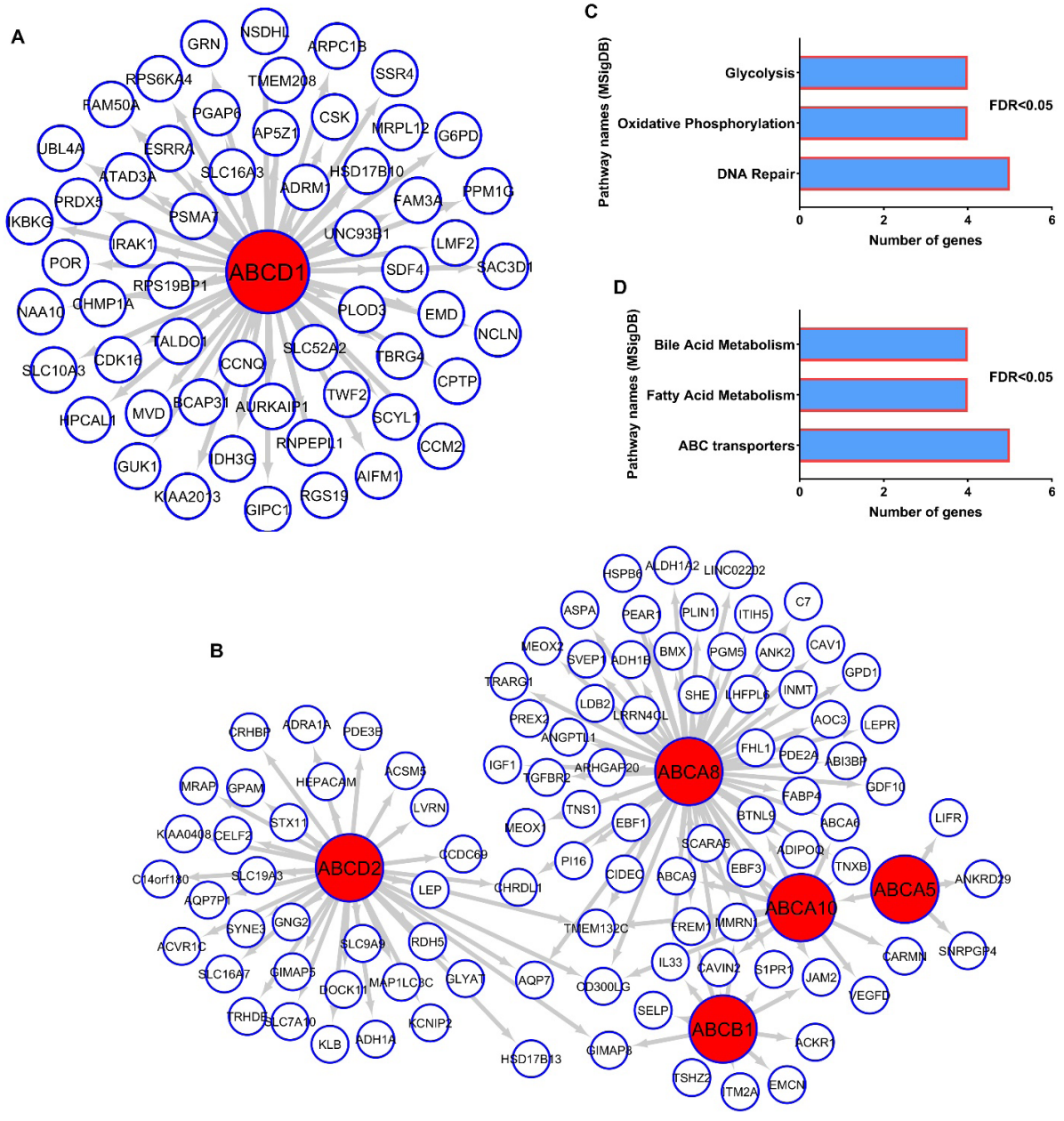

Figure 3. Co-expression of candidate ABC family genes with genes associated with the malignancy pathway

A and B) The co-expression network for candidate genes based on TCGA data, (C) The outcomes of the pathways linked to the co-expression network for *ABCD1*, D) Pathways associated with the genes in the co-expression network, as shown in B

sistance to Docetaxel was linked to elevated expression of *ABCA5*, *ABCA8*, *ABCA10*, and *ABCB1* (Figure 5). Notably, the preceding section demonstrated that the luminal A subgroup exhibited higher levels of expression for *ABCA5*, *ABCA8*, *ABCA10*, and *ABCB1* compared to other subgroups. These findings suggest that the elevated expression of the aforementioned genes may be the source of Docetaxel resistance in the luminal A subgroup. However, these findings are merely a preliminary indication, and additional verifications, such as in vitro and in vivo research, are required.

Discussion

This study found that eight genes (*ABCA12*, *ABCA3*, *ABCB8*, *ABCB9*, *ABCC5*, *ABCD1*, *ABCG1*, *TAP1*) have increased expression, while 19 genes (*ABCA1*, *ABCA10*, *ABCA13*, *ABCA4*, ABCA5, *ABCA6*, *ABCA8*, *ABCA9*, *ABCB1*, *ABCB11*, *ABCB5*, *ABCC2*, *ABCC6*, *ABCC9*,

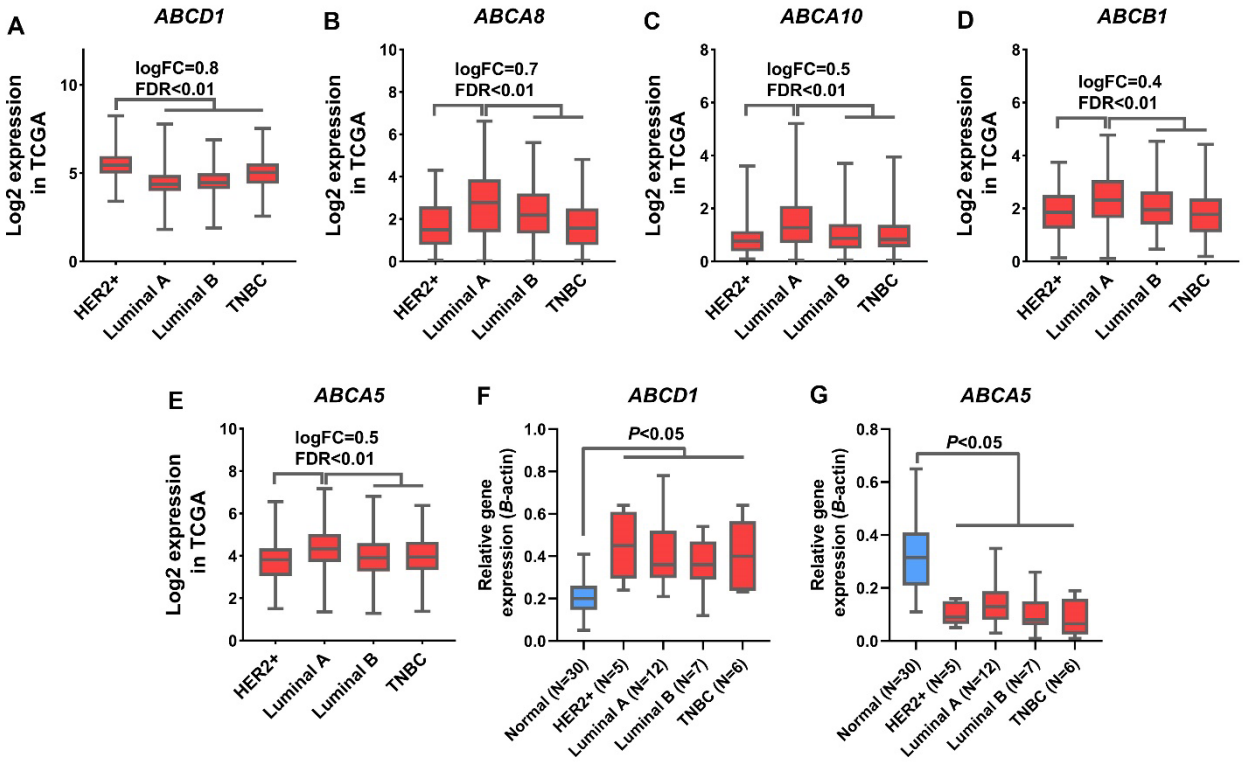

Figure 4. A-E) The expression of candidate genes in different BC subgroups based on TCGA data, F and G) The results of RT-qPCR were determined for the expression levels of *ABCD1* and *ABCA5* in different BC subgroups

Note: The results showed increased expression of ABCA5 and decreased expression of *ABCD1*.

ABCD2, *ABCD4*, *ABCG2*, *CFTR*) have decreased expression. Prognosis was positively correlated with *ABCD1* and the downregulated genes: *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*. Six genes from the ABC family (*ABCD1*, *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*) are potentially linked to BC malignancy and may serve as predictive biomarkers. Downregulation of 19 ABC transporter genes in BC may contribute to tumor malignancy through multiple mechanisms. Many of these transporters, such as *ABCA1*, *ABCB1*, and *ABCC2*, are involved in the efflux of toxic compounds and metabolites; reduced expression can lead to their intracellular accumulation, oxidative stress, genomic instability, and enhanced tumor growth [9, 20, 21]. Others, including *ABCA1*, *ABCA5*, and *ABCG2*, regulate lipid metabolism and cholesterol transport, where diminished activity may alter membrane composition, disrupt lipid-dependent signaling, and promote migration and invasion [9, 21, 22]. Additionally, reduced expression of certain ABC transporters may impair antigen presentation and modulate the tumor microenvironment toward hypoxia and inflammation, collectively fostering BC progression [23]. A 2013 study on ABC transporter gene expression found *ABCA5/6/8/9/10*, *ABCB1/5/11*, *ABCC6/9*, *ABCD2/4*, *ABCG5*, *ABCG8* significantly downregulated in post-treatment tumors, while *ABCA2/3/7/12*, *ABCB2/3/8/9/10*, *ABCC1/4/5/10/11/12*, *ABCD1/3*, *ABCE1*, *ABCF1/2/3*, and *ABCG1* were upregulated. Post-treatment response of BC patients to neoadjuvant chemotherapy is linked to *ABCA12*, *ABCA13*, and *ABCD2* [24].

However, compared to normal samples, the expression levels of *ABCD1*, *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2* showed good sensitivity and specificity in cancerous samples. This suggests that candidate genes derived from the ABC family may be more significant in the pathophysiology of BC than other family members.

The analysis of the *ABCD1* co-expression network revealed that 54 genes have an expression connection with the gene's expression level; some of these genes are highly implicated in pathways, such as glycolysis, oxidative phosphorylation, and DNA repair. Numerous facets of DNA repair mechanisms have been linked to ABC transporters [25]. They are known to play a role in identifying and repairing DNA damage, especially when specific ABC proteins, such as the MutS ATPase,

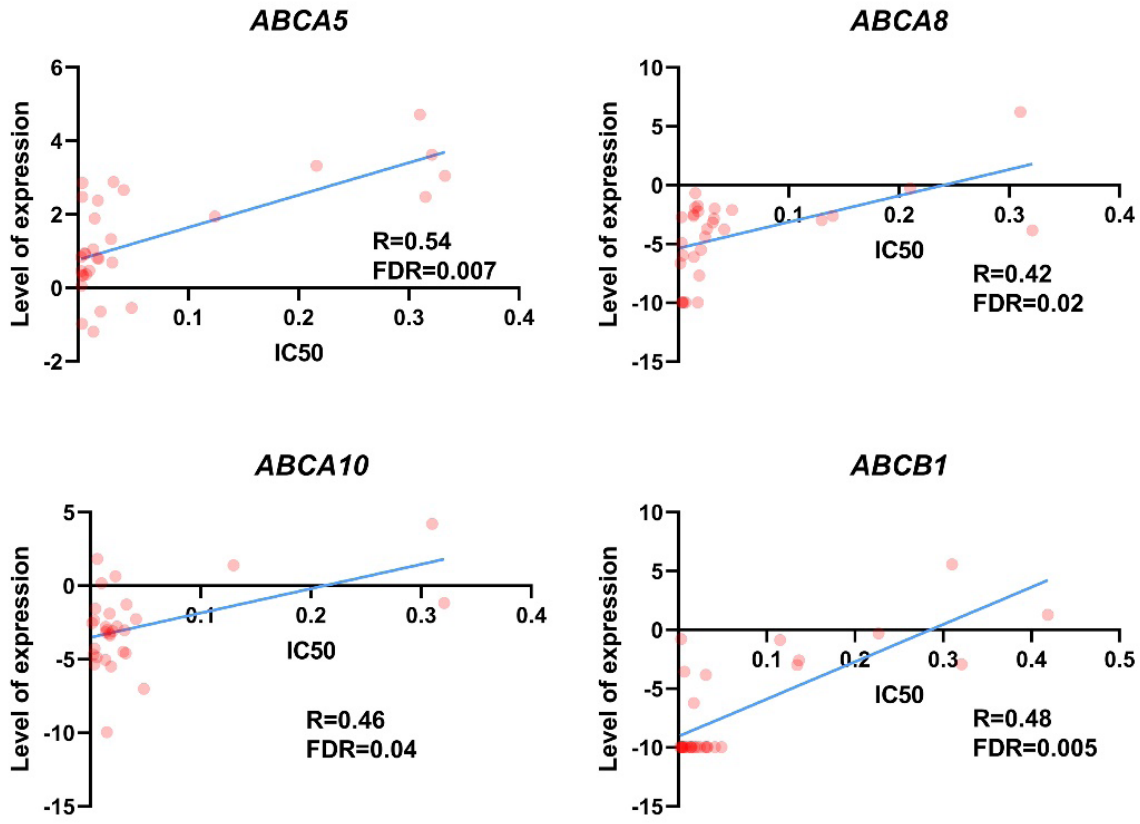

Figure 5. Using GRAY and UHNBrest data, the association between drug resistance and sensitivity and potential gene expression was examined

Note: Interestingly, drug resistance to Docetaxel was linked to elevated expression of *ABCA5*, *ABCA8*, *ABCA10*, and *ABCB1*.

a mismatch repair system component, are active [26]. To identify and bind to mispaired DNA bases and facilitate their repair, this protein utilizes ATP [22]. Furthermore, by mediating the efflux of hazardous substances from cells, specific ABC transporters, including *ABCB1* (P-glycoprotein), support cellular resistance to genotoxic chemicals [27]. Studies have demonstrated that increased *ABCB1* expression in bat cells protects the cells from DNA damage caused by genotoxic medications, which may help explain why bats have a low incidence of cancer [28]. Additionally, iron-sulfur clusters are critical cofactors for many mitochondrial enzymes involved in oxidative phosphorylation [29], and *ABCB7* is necessary for their biosynthesis. Heme biosynthesis involves *ABCB10*, which also protects against oxidative damage caused by heme metabolism [30, 31].

However, the current study identified 122 genes associated with the expression levels of *ABCA10*, *ABCA5*, *ABCA8*, *ABCB1*, and *ABCD2*. According to the pathway analysis results, these 122 genes were involved in pathways, such as ABC transporters, fatty acid metabolism, and bile acid metabolism. Previous studies have demonstrated that *ABCD1* is crucial for the oxidation of very long-chain fatty acids (VLCFAs) and that mutations in this gene lead to X-linked adrenoleukodystrophy, characterized by the accumulation of VLCFAs resulting from impaired peroxisomal β-oxidation [32]. While ABCD3 has been shown to transport branched-chain fatty acids and bile acid intermediates, suggesting its involvement in both fatty acid and bile acid metabolism, *ABCD2* also transports acyl-CoA derivatives [32]. Additionally, *ABCA1* and other members of the ABC subfamily help maintain lipid homeostasis by promoting the efflux of phospholipids and cholesterol, which, in turn, indirectly affects fatty acid metabolism by altering the cellular lipid composition [33].

According to the gene expression analysis findings, the HER2+ subgroup exhibited a higher level of *ABCD1* gene expression than the other subgroups. Conversely, the luminal A subgroup exhibited higher expression

levels of *ABCA8*, *ABCA10*, *ABCA5*, and *ABCB1* compared to different subgroups. There was no evidence of *ABCD2* expression reliance among the various subtypes of BC. Additionally, according to the qRT-PCR data, there was a substantial decrease in the expression level of ABCA5 in cancerous samples compared to healthy samples and an increase in the expression of ABCD1 in cancerous samples compared to healthy samples. Studies on colorectal cancer have shown that patients with lower levels of *ABCA5* have worse overall survival outcomes. This suggests that *ABCA5* may protect against tumor progression, and its downregulation may increase the aggressiveness of the cancer [34]. In additional research, immunohistochemical tests revealed that the normal tissues of human renal cell carcinoma had significantly higher levels of *ABCD1* expression than their malignant counterparts [35].

The findings of the current study regarding drug resistance indicate that resistance to Docetaxel is linked to elevated expression levels of *ABCA5*, *ABCA8*, *ABCA10*, and *ABCB1*. Consistent with these findings, research has demonstrated that overexpression of *ABCB1* is frequently associated with resistance to various anticancer medications, including Docetaxel, an antimitotic agent primarily used to treat breast and prostate cancers [36, 37]. Another limitation of the study is the lack of in vitro testing.

Conclusion

According to the study's findings, variations in the expression of ABC family genes in BC may serve as prognostic indicators. In particular, BC malignancy is associated with decreased expression of *ABCA5*, *ABCA10*, and *ABCA8* and increased expression of *ABCD1*. Furthermore, more research is necessary to determine the relationship between the expression of these genes and other BC subtypes, as well as treatment resistance to Docetaxel. Nonetheless, our results suggest that these genes play a crucial role in the etiology of treatment resistance and BC. Finally, the need for further research to validate and elucidate these findings is emphasized.

Ethical Considerations

Compliance with ethical guidelines

The review board at Imam Khomeini Hospital examined all bioethical issues and confirmed compliance with the guidelines established by Iran's Ministry of Health and Medical Education. Written consent was obtained from every individual involved in the study. This syudy was approved by the Research Ethics Committee of Payam Noor University, Tehran, Iran (Code: IR.PNU.REC.1403.365).

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Authors contribution's

Study design: Mohammad Parishani and Mohammad Mahdevar; Experiments, data analysis, and writing the original draft: Mohammad Parishani; Data interpretations: Mohammad Fazilati, Habibollah Nazem, and Hossein Salavati; Bioinformatics analysis and final approval: Mohammad Parishani and Mohammad Mahdevar.

Conflict of interest

The authors declared no conflict of interest.

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