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ORIGINAL ARTICLE

Expression of genes related to iron homeostasis in breast cancer

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Abstract

Background The dysfunctions in the metabolism of iron have an important role in many pathological conditions, ranging from disease with iron deposition to cancer. Studies on malignant diseases of the breast reported irregular expression in genes associated with iron metabolism. The variations are related to findings that have prognostic significance. This study evaluated the relationship of the expression levels of *transferrin receptor 1* (*TFRC), iron regulatory protein 1* (*IRP1*), *hepcidin* (*HAMP*), *ferroportin 1* (*FPN1*), *hemojuvelin* (*HFE2), matriptase 2* (*TMPRSS6*), and *miR-122* genes in the normal and malignant tissues of breast cancer patients.

Methods & Results The normal and malignant tissues from 75 women with breast malignancies were used in this study. The patients did not receive any treatment previously. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used in figuring the levels of gene expression associated with iron metabolism. When the malignant and normal tissues gene expression levels were analyzed, expression of *TFRC* increased (1.586-fold); *IRP1* (0.594 fold) and *miR-122* (0.320 fold) expression decreased; *HAMP*, *FPN1*, *HFE2*, and *TMPRSS6* expressions did not change. *FPN1* and *IRP1* had a positive association, and this association was statistically significant ($r = 0.266$; $p = 0.022$). *IRP1* and *miR-122* had a positive association, and this association had statistical significance ($r = 0.231$; $p = 0.048$).

Conclusions Our study portrayed the important association between genes involved in iron hemostasis and breast malignancy. The results could be used to establish new diagnostic techniques in the management of breast malignancies. The alterations in the metabolism of malignant breast cells with normal breast cells could be utilized to achieve advantages in treatment.

Keywords Iron homeostasis genes · Breast cancer · Gene expression · TFRC · IRP1 · miR-122

Introduction

Malignancies of breast cancer account for 12.5% of the newly diagnosed malignancies, globally, making it the most commonly seen cancer [1]. The etiology of breast cancer is multifactorial. Lifestyle factors, environmental factors, and genetic factors are related to malignant transformation [2].

Iron has major functions in the metabolism of many organisms. However, the exuberance of iron could lead to cellular injury by free radicals. Disorders of iron metabolism are associated with many diseases and malignant transformation. Cellular proliferation and tumorigenesis are linked with iron metabolism. Bogdan et al. showed the elevation in cellular iron levels in malignant tissues, caused by disruption of the metabolism of iron [3]. Silva B. et al. showed the presence of various mechanisms in iron homeostasis and the

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relationship between these mechanisms have major significance [4].

The homeostasis of iron is maintained by controlling its absorption, mobilization of iron storage, and recycling. Iron is transported in the blood by transferrin. It has transferrin receptor protein 1 (TfR1) on the surface of the cell, which is a specific receptor. TfR1 is coded by the *transferrin receptor 1* (*TFRC*) gene. Cytoplasmic proteins, Iron regulatory proteins 1 & 2 (IRP-1 & IRP-2) [5], also called cellular "iron sensor proteins", control the translation of genes responsible for iron and energy metabolism. The decrease in the intracellular iron levels leads to the activation of IRP1, which binds to TfR mRNA and its stabilization, leading to the decrease of the translation. Increases in the levels of iron in the cell lead to the inhibition of the iron-responsive elements (IRE)-binding capacity of IRP1 [6].

Hepcidin is a peptide hormone, formed by 25 amino acids. *Hepcidin antimicrobial peptide* (*HAMP*) gene has the genetic code for Hepcidin. It is secreted by the hepatocytes, and it plays a role in the tissue distribution and absorption of iron [7]. Iron absorption in the intestinal tract, plasma iron concentrations, and distribution of iron to the tissues is controlled by hepcidin. This process starts with the initiation of the deterioration of the receptor. Ferroportin (*FPN1*; *ferroportin-1* or *SLC40A1 gene*) has a major part in the transportation of iron between cells and blood. The linking of hepcidin to ferroportin starts its internalization, consequently, its degradation; giving was to the increase in the levels of iron intracellularly [8].

Hemojuvelin (HJV) is one of the main components of the BMP-SMAD signaling pathway. The rise of iron stores triggers hepcidin expression upregulation [9]. *Hemochromatosis type 2 protein* (*HFE2*) gene encodes HJV. Matriptase 2 is encoded by the *Transmembrane serine protease 6* (*TMPRSS6*) gene. BMP co-receptor HJV which is located in the BMP-SMAD pathway is cleaved by the Matriptase 2. This helps control the expression of hepcidin. miRNAs, especially miR-122, have been reported to control hemojuvelin levels [9]. miR-122 acts on mRNA expression of hemojuvelin, in the liver tissue, which is a major part of controlling the tissue iron levels [10].

This study aimed to compare the levels of expression of *TFRC, IRP1, HAMP, FPN1, HFE2, TMPRSS6*, and *miR-122* genes in the normal and malignant tissues of patients with breast cancer.

Materials and methods

Seventy-five female patients with primary breast cancer who were operated on before any form of neoadjuvant treatment were involved in this study. The mean age was 54 (± 13 years). The inclusion criteria were the presence of primary breast malignancy. The exclusion criteria were administration of any neoadjuvant treatment (radiotherapy and/or chemotherapy and/or endocrine therapy) presence of other cancers, recurrent breast cancer, and unavailability of clinical data. Istanbul University-Cerrahpasa Cerrahpasa Medical School Institutional Review Board approved the study. It was performed based on the principles of the Declaration of Helsinki. Informed consent was acquired from all of the participants.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The tissue samples were taken from the tumor and the normal tissues outside the tumor microenvironment. PureLink® RNA Mini Kit (Thermo Fisher Scientific, USA) was utilized for the isolation of the total RNA. NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) was used to find the levels of the total RNA. The level of the total RNA calculation was used for the equalization of the initial RNA used for cDNA synthesis. TranScriba Kit (A&A Biotechnology, Poland) was utilized for the cDNA synthesis. StepOnePlus Real-Time PCR System appliance (Applied Biosystems, USA) was utilized for qPCR analyses. TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, USA) and Taqman® Gene Expression Assays (Thermo Fisher Scientific, USA) were used for the qPCR analysis (Tables 1 and 2). The recommendations from the manufacturer were followed. The process started at 50 °C for two minutes. The initial denaturation-hot start at 95 °C (Holding Stage) for 10 min, accompanied by 40 cycles of the two-step reaction (Cycling Stage). The two-step reaction was 10 s at 95 °C and sixty seconds at 60 °C. The expression studies for the genes were done in triplicate.

Statistical analysis

Quantitative-Comparative Ct (ΔΔCт) method was used for finding the gene expression levels. $2^{-\Delta\Delta C_T}$ values of the target genes were calculated by Cycle Threshold (CT) values of Actin, beta gene (*ACTB)*. ACTB gene was used as a housekeeping gene. *miR-122*'s relative expression was determined by the utilization of *RNU44* as the housekeeping gene. the $2^{-\Delta\Delta C_T}$method was used to observe the relative changes in the target and the housekeeping gene expression in the normal tissue samples and the malignant tissue samples [11]. Mean+standard deviation (SD) was used for the age and mean+error (SE) was used for the gene expression, in the expression of the continuous values. $p < 0.05$ was accepted as the statistical significance level. Mann-Whitney test was used for the analysis of the two groups in

Table 1 TaqMan mRNA Assay ID numbers

Assay ID	Gene	RefSeq	Exon Boundry	Amplicon length (bp)
GENEXs300	TMPRSS6	NM_001289001.2	11–12	87
		NM_001289000.2	11–12	87
		NM_153609.4	11–12	87
		NM_001374504.1	11–12	87
GENEXs300	HFE2	NM_213653.4	4–4	89
		NM_001379352.1	4–4	89
		NM_145277.5	3–3	89
		NM_001316767.2	4–4	89
		NM_202004.4	3–3	89
		NM_213652.4	2–2	89
GENEXs300	HAMP	NM_021175.4	1–3	101
GENEXs300	IRP1	NM_001278352.2	20–21	75
		NM_001362840.2	20–21	75
		NM_002197.3	19–20	75
GENEXs300	FPN1	NM_014585.6	5–6	76
GENEXs300	TFRC	NM_003234.4	9–10	82
		NM_001128148.3	9–10	82
		NM_001313965.2	8–9	82
		NM_001313966.2	7–8	82
GENEXs300-C	ACTB	NM_001101.5	4–5	114

Table 2 TaqMan miRNA Assay ID numbers

Gene ID	Accession No	Assay Name and ID No	Amplicon length (bp)
hsa-miR-122-5p	MIMAT0000421	miRNA qPCR SL Assay for hsa-miR-122-5p, MIREX-H122	62–66
hsa-RNU6-6P	Entrez Gene ID: 26,826	miRNA qPCR U6 Control Assay for Human RNU6-6P, MIREX-U6	89

RT-qPCR, for statistical analysis. The relationship between gene expressions was analyzed by the utilization of Pearson correlation. The statistical analysis was done by statistical software package (SPSS, v18.0, SPSS Inc., Chicago, IL).

Results

The demographical and histopathological data of the patients are provided in Table 3. In this study, the expression levels of *TFRC, IRP1, HAMP, FPN1, HFE2, TMPRSS6*, and *miR-122 genes* in the malignant tissues and the normal tissue samples near the malignant tissues from 75 female patients were determined (Table 4).

Table 3 Demograhic data of the breast cancer patients

Age:	47		
≥ 50	28		
< 50			
ER	61	**Tumor location**	34
Positive	14	Right breast	41
Negative		Left breast	
PR	55	**Nuclear stage**	41
Positive	20	I,II	34
Negative		III,IV	
HER-2	14	**Histological stage**	47
Positive	61	I,II	28
Negative		III,IV	
Triple negative status	6	**Tumor Size**	33
Pozitif	69	< 2	42
Negatif		> 2	
Lymph node metastasis	43	**Clinical stage**	51
Metastasis	32	I,II	24
No metastasis		III,IV	
Metastatic status	3	**Microcalcification**	57
Metastatic	72	Present	18
Non-metastatic		Not present	
p53 status	23	**Histological type**	61
Positive	52	Ductal	7
Negative		Lobular	7
		Other	

The expression levels of *TFRC* in the malignant tissues were 1.586 fold increased in comparison with the normal tissues based on $2^{-\Delta\Delta C_T}$ calculations. The expression levels of *TFRC* in the malignant tissues (0.0115 ± 0.0012) were increased when compared with the normal tissue expression levels of *TFRC* (0.0062 ± 0.0005)($p = 0.0001$). On the other hand, the expression levels of *IRP1* in the malignant tissue were found to be 0.594-fold decreased compared to the normal tissue. The expression levels of *IRP1* in the malignant tissue (0.0034 ± 0.0003) were lower than the expression levels of *IRP1* in the normal tissue (0.0055 ± 0.0004), and this difference was statistically significant ($p = 0.0001$). Also, the *miR-122* expression levels in tumor tissue were found to be 0.32-fold decreased when compared to the normal tissue. The expression levels of *miR-122* in the malignant tissue (0.0003 ± 0.0002) were 0.32 fold decreased than its expression levels in the normal tissue (0.0008 ± 0.0001). This difference had statistical significance ($p = 0.025$).

The expression levels of *TMPRSS6* in the malignant tissues (0.0190 ± 0.0121) were 1.912 fold increased than the normal tissue (0.0133 ± 0.0029). Nonetheless, the difference was not statistically significant ($p = 0.651$). The expression levels of *HFE2* in the malignant tissues (0.0228 ± 0.0080) were 0.531-fold decreased than the expression levels in the normal tissue(0.0312 ± 0.0050). The difference did not reach statistical significance ($p = 0.368$). The expression levels of *HAMP* in the malignant tissue (0.0057 ± 0.0012) were 1.34-fold increased than the expression levels in the normal tissue(0.0055 ± 0.0011), however, the difference was

Table 4 Expression values and fold changes of genes in tumor and normal tissue samples

Gene	N	Group	ΔCtb (Mean ± SE)	$2^{-\Delta CT}$ (Mean ± SE)	ΔΔCtb (Mean ± SE)	Fold change	P-value
FPN1	75	N	5,2523± 0,1092	0,0331± 0,0028	0,6733± 0,2027	0,627	0,836
		T	5,9256± 0,1992	0,0319± 0,0051			
HAMP	75	N	8,8239± 0,2385	0,0055± 0,0011	0,0488± 0,1928	1,034	0,911
		T	8,8573± 0,2276	0,0057± 0,0012			
HFE2	75	N	6,5880± 0,2838	0,0312± 0,0050	0,9129± 0,2102	0,531	0,368
		T	7,5009± 0,2698	0,0228± 0,0080			
IRP1	75	N	7,7353± 0,1104	0,0055± 0,0004	0,7507± 0,1039	0,594	0,0001
		T	8,4861± 0,1065	0,0034± 0,0003			
TFRC	75	N	7,6217± 0,1061	0,0062± 0,0005	0,6658± 0,1381	1,586	0,0001
		T	6,8808± 0,1448	0,0115± 0,0012			
TMPRSS6	75	N	9,3419± 0,3254	0,0133± 0,0029	0,9353± 0,3720	1,912	0,651
		T	8,4066± 0,3308	0,0190± 0,0121			
miR-122	75	N	2,5377± 1,3422	0,0008± 0,0001	0,0607± 0,7548	0,32	0,025
		T	2,7238± 1,4169	0,0003± 0,0002			

SE: Standart error

not statistically significant (p = 0.911). The *FPN1* expression levels in the tumor tissue were found to be 0.627-fold decreased when compared to the normal tissue. The expression levels of *FPN1* in the malignant tissue (0.0319 ± 0.0051) were 0.627 fold decreased than the expression levels in the normal tissue (0.0331 ± 0.0028), and the difference did not reach statistical significance (p = 0.836).

According to the calculation of $2^{-\Delta\Delta C_T}$, the 0.9–1.1 range was used for the evaluation. When the value was lower than 0.9, the gene expression was accepted to be less than the expression in the normal tissue. 0.9–1.1 range was accepted as not changed in comparison with the expression in the normal tissue. 1.1 and values above this level were defined as increased gene expression in comparison with the normal tissue. *TFRC* gene expression was found to be elevated in 50 (67%), and reduced in 17 (23%) tissues. The *TFRC* gene expression was not altered in 8 (10%) tissues. IRP1 gene expression was elevated in 8(11%), reduced in 58 (77%) tissues; and was not altered in 9 (12%) tissues. The *miR-122* gene expression was elevated in 12 (16%), reduced in 62 (83%) tissues; and not altered in 1 (%1) tissue. The *TMPRSS6* gene expression elevated in 46 (61%), reduced in 26 (35%) tissues and it was not altered in 3 (4%) tissues. The *HFE2* gene expression was elevated in 16 (21%), reduced in 50 (67%) tissues and it was not altered in 9 (12%) tissues. The *HAMP* gene expression was elevated in 35 (47%), reduced in 30 (40%) tissues, and it was not altered in 10 (13%) tissues. The *FPN1* gene expression was elevated in 27 (36%), reduced in 42 (56%) tissues; and it was not altered in 6 (8%) tissues.

In our study, the relationship between *TFRC, IRP1, HAMP, FPN1, HFE2, TMPRSS6*, and *miR122* expression levels was evaluated using the Pearson correlation test. Two statistically significant relationships were unraveled. These were *IRP1* and *FPN1* (r = 0.266; p = 0.022), and *IRP1* and *miR-122* (r = 0.231; p = 0.048).

Discussion

Increased levels of cellular iron are vital for the growth and rapid proliferation of cancer cells. Various studies portrayed the importance of the dysregulation of proteins enrolled in iron metabolism, which have vital parts in malignant transformation. Alterations of genes related to iron hemostasis had been thought to be prognostic biomarkers and therapeutic targets in malignancies. Miller et al.'s study reported that attrition of genes related to iron excretion and in addition to this the upregulation of the iron uptake process had effects on the prognosis of breast cancer [12].

The *TFRC* gene encodes TfR1, a receptor located on the cell surface, essential for iron absorption into the cell. TfR1 had increased expression in cells with rapid proliferation, like cancer cells, which have a high demand for iron during proliferation. Based on this knowledge, the upregulation of the *TFRC* gene had been defined as a target candidate in cancer therapy. Increased expression of this gene was found to be associated with cancer cell proliferation and poor survival in patients with breast malignancy, in a report by Habashy et al. [13]. Jiang et al. reported the expression of *TFRC* was elevated in malignant breast cells (MCF-7 cells) than cells in the normal mammary glands(MCF-12 A cells) [14]. In alignment with these prior findings, in our study, the expression levels of TFTC were significantly upregulated in the malignant breast cells.

Transcriptional, and post-transcriptional processes affect the TFRC expression. The iron-responsive element system (IRP1/2-IRE), controls the iron homeostasis through

post-transcriptional modulation of the TFRC through a sophisticated system. Chen et al. showed that deprivation of iron led to the binding of IRP1/2 to IREs, which led to the stabilization of the mRNA of TFRC, thus raising the expression of proteins [15]. Wang et al. showed that induced *IRP1* expression in malignant breast cells (MCF7) resulted in the elevation of mRNA and protein levels of TFRC [16]. The expression level of the *IRP1* gene was reduced in the malignant cells in our study. Therefore, we cannot postulate the relationship between the elevation of the expression of *TFRC* and expression levels of the *IRP1* gene. Wang et al. showed that overexpression of *IRP2* was associated with reduced ferritin heavy chain and TfR1 elevation. However, this relationship was not present for *IRP1*. Wang et al., reported that *IRP2* expression alone was associated with the molecular subtype and the histological grade of human breast cancer [16]. Increased levels of TfR1 expression were reported in prostate cancer cells [17]. In a study by Deng et al., the expression level of *IRP2* was detected in 4 malignant prostate cell lines, and *IRP1* expression level had a moderate rise in only 2 cell lines [17]. The increase in the regulation of *IRP2* in malignant prostate cells homogenizes normal iron control pathways, which gives way to an elevation in the levels of iron, aiding tumor proliferation. *IRP1* was reported to be downregulation in hepatocellular carcinoma, and this could be used to predict the stage of the tumor and the prognosis [18]. Oxygen, oxidative stress, and the levels of nitric oxide are among the additional molecules and processes associated with the response of IRPs in addition to cellular iron levels. It was reported by Luo et al. that hypoxia in the short-term lead to a decline in mRNA of IRP1and levels of protein in HepG2 cells [19].

The most essential iron efflux transporter is ferroportin protein (FPN1). Its function is the siphonage of the iron to the cytoplasm of the macrophages and the enterocytes. FPN1 is encoded by the *SLC40A1* gene. Its expression is controlled by hepcidin, encoded by the *HAMP* gene [20]. The dysregulated hepcidin–FPN1 signaling was related to a rise in malignant transformation by experimental and epidemiological studies. However, the exact processes associated with the dysregulation of iron-related gene expression in the malignant processes are still not uncovered. In our study, no difference in the hepcidin gene expression was identified. On the other hand, a statistically insignificant decrease in the *FPN*1 gene expression was seen. The rise in hepcidin levels was seen in the lung, breast, prostate, and renal cancer in addition to various other cancers. However, its levels were reduced in some brain tumors and hepatocellular carcinoma [21]. Shen et al. reported the findings in hepatocellular carcinoma, where the levels of hepcidin were reduced in malignant cells than the normal cells [22]. In breast cancer, Zhang et al. reported limited elevations in the expression levels of hepcidin in malignant cells than the normal tissues [23]. Similar to our study, the tumor ferroportin concentration was found to be low in the breast tumors in that study. In addition, Pinnix et al.'s study revealed that the mRNA level of tumor hepcidin was able to discern only marginal significance for patient prognosis [24]. The major and most prominent hepcidin expression simulators are bone morphogenetic proteins (BMPs). Reports have shown that BMP6 was not alone, other BMPs also regulate hepcidin expression [21]. The UniGene databases in silico analysis showed that malignant breast tissue had reduced expression of ferroportin [25]. Our study showed that ferroportin gene expression in the cancer tissue was also found to be 0.627 times reduced compared to the normal tissue.

The coreceptor of BMP, hemojuvelin's cleavage blocks the BMP-SMAD signaling pathway, through inhibition of the expression of hepcidin by matriptase-2 [26]. In our study, matriptase-2 expression increased (1,912-fold) and hemojuvelin gene expression decreased (0.531-fold) in the malignant breast tissues. In our previous study that we conducted on breast cancer patients, the levels of the expression of the *TMPRSS6* gene in the malignant tissues were 1.88-fold elevated than normal tissues [27]. Normal breast tissue samples were reported to have dominant matriptase-2 expression [28]. Overall et al.'s study showed that the gene expression levels of *TMPRSS6* were elevated in the invasive ductal carcinoma cells [29]. Gitlin-Domagalska et al. showed the relationship of *TMPRSS6* with a decline in gene expression levels, progression of tumor cells, and poor prognosis [30].

We could not find any literature investigating homojuvenile gene expression in breast cancer. The simulation of hepcidin transcription by BMP is inhibited by matriptase-2. This process is achieved by the cell surface proteolytic processing of the BMP co-receptor hemojuvelin [28]. Controversial results had been reported on BMP's role in malignancies of the breast. The BMP6's role in breast malignancies was variable according to several studies, performed on the relationship between hemojuvelin and BMP6. Most of the reports present that BMP6 expression is reduced in malignant breast tissue [31]. Our study showed that the gene expression of *TMPRSS6* elevated in the malignant tissues. A hypothesis could be constructed on the interaction between matriptase-2 with the BMP6/SMAD signaling via hemojuvelin. The low homojuvenile expression we found in our study supported this hypothesis.

Hepatic *miR-122* expression had paramount importance in sustaining the iron levels in the liver and the plasma. Castoldi et al. reported that the inhibition of the expression of hepatic *miR-122* in the liver tissue of mice and primary murine hepatocytes, lead to a rise in mRNA expression of hepcidin and homojuvenile [10]. We portrayed that *miR-122*

expression was reduced in the malignant breast tissues. On the other hand, in our study, we found a decrease in the expression of homojuvenile in malignant breast tissues, while the hepcidin expression was unchanged. miR-122 was reported to be essential in the regulation of serum cholesterol levels [32]. Therefore, the overlapping or non-overlapping metabolic pathways in which miR-122 plays a role may alter the effects of mir-122 on gene expressions. On the other hand, various studies studied the role of mir-122 in malignant breast tissue. Similar to our study, Yan et al. reported that *miR-122* levels were reduced and decreased in malignant breast cells than the normal tissue samples; and this finding was statistically significant [33]. Two studies portrayed the *miR-122* downregulation in malignant breast cells [34, 35]. Several other studies showed that the expression of *miR-122* elevated in malignant breast tissue more than the normal tissue [36].

Several studies have addressed the relationship between the gene expression levels used in this study and the clinicopathological characteristics of patients [37, 38]. We believe that analyzing our data according to these clinical data would lengthen our study, thus we plan to do this analysis in a future study.

In conclusion, our study shows a powerful association between genes enrolled in the metabolism of iron and breast cancer, paving the way for the establishment of new tools to be used for the prognosis of breast cancer. They could also aid in the establishment to find new methods to identify normal and malignant breast cells, which could be utilized for therapeutic advantage.

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Data Availability The data of the study are available upon request from the corresponding author.

Declarations

Compliance with ethical standards The authors declare no conflict of interest.

The ethical approval was obtained from Istanbul University-Cerrahpasa Cerrahpasa Medical School Institutional Review Board on June 6th, 2016 with the serial code 214246. All of the procedures on human participants were in accordance with the declaration of Helsinki in 1964, its amendments, and parallel ethical standards.

All the patients in this study provided informed consent before inclusion.

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