



ROLE OF MIRNA-122 AND MIRNA-200B IN INTRATUMOR HETEROGENEITY FORMATION AND HUMAN BREAST CANCER PROGNOSIS

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ABSTRACT

Aim: To determine the features of miR-122 and -200b expression signature in BC patients due to major clinical-pathological characteristics of the disease.

Methodology: The expression levels of miR-122 and -200b and ER, PR, Her2/neu, Ki-67, E-cadh, N-cadh, FTH1, Hspc were analyzed in cancer tissue and sera of BC patients. Relative expression levels of the miR-122 and -200b were examined using *qRT-PCR* (Quantitative Reverse Transcription *PCR*), *protein expression was measured by immunohistochemical analysis*.

Results: Correlation between miR-122 and -200b expression clinical-pathological characteristics of BC was established. Prognostic value of miR-122 and -200b was estimated.

Discussion and Conclusion: Changes of miR-122 and -200b expression in tumor tissue and sera of BC patients provide information about major clinical-pathological characteristics of BC.

Key Words: miRNA, Breast cancer, Prognosis

Abbreviations used: Breast cancer (BC), miRNA (miR).

INTRODUCTION

Intratumor heterogeneity is considered to be characteristic of most malignant tumors and is the main obstacle to effective treatment. Exploration of intratumor heterogeneity, mechanisms of its formation are in the number of the urgent fields of fundamental oncological research. The biological phenomenon of intratumor heterogeneity, based on the genetic and epigenetic instability, is regarded to be a key factor that determinates tumor development from of its origin, to implementing of various pathways of tumor progression, its aggressiveness.

According to many researchers, intratumor heterogeneity plays a crucial role in the rate of tumors, supporting its oncogenic potential, cell survival in a dynamic microenvironment. Manifestation of existing heterogeneity within a tumor are morphological structure, genetic, epigenetic status differences in cells populations, as well as the variability of expression of different molecular markers. [1].

MiRNAs play an important role in the formation of cell diversity inside the tumor. MiRNAs are noncoding class of small RNAs that regulate the expression of almost a third of all genes at posttranscriptional level [2]. MiRNAs play a key role in maintaining of cellular homeostasis and are involved in regulation of cell cycle, differentiation, processes of inflammation, apoptosis and invasion [3]. At the same time, miRNAs can act as a paracrine and autocrine regulators of biological behavior of tumor microenvironment. According to the literature the origin and progression of several tumors may be due to changes in specific miRNAs expression [4].

Different miRNAs can either stimulate or inhibit tumor development and metastasis, and increase sensitivity or resistance to chemotherapy, thereby acting as tumor suppressors or oncogenes. It is known that the level of miRNAs correlates with vascular invasion and proliferation. Numerous studies demonstrate the possibility of using the expression levels of tissue-specific miRNAs as diagnostic biomarkers for prognosis and response evaluation to therapy [6, 7].

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Important advantage of miRNAs over using other known markers is that unlike screening of expression of a large number of genes, it is enough to analyze a small number of miRNAs. As in plasma and in paraffin blocks miRNAs remain stable [8].

The development of tumors of different histogenesis is accompanied by changes in levels of circulating and tumor miRNAs, which are specific to certain tumors localization.

For today several dozens of miRNAs that may be potential markers of breast cancer were studied. But changes in the concentration of many miRNAs are the same for different types of malignancies and their molecular subtypes. Therefore, it is necessary to search for miRNAs that are specific for breast cancer and each of its subtype.

The fact that miR-122 and 200b are involved in breast cancer carcinogenesis, and act as oncosuppressors is well-known, but their role in tumor prognosis remains ultimately undefined.

Therefore the aim of this study was to investigate the participation of miR-122 and -200b in the shaping of intratumor heterogeneity and prognosis of human breast cancer.

MATERIAL AND METHODS

A total of 134 subjects were recruited for this study of which 120 were those who suffering from breast cancer, 14 subjects included age-matched healthy individuals.

120 tumor samples and 14 samples of normal breast tissue obtained during surgery and 120 samples of blood serum of patients with breast cancer and 14 blood serum samples of healthy donors were studied. Tumor stage was determined by TNM staging system (2008). The histological type of tumor verified at the morphological study of paraffin-embedded tissue according to the WHO (2006). All patients before surgery received neither radiation nor chemotherapy. All patients were examined using conventional clinical and laboratory methods according to the standards of diagnosis and treatment of cancer patients, approved by the Ministry of Health of Ukraine №554 from 17.09.2007.

All patients and donors were informed and agreed to the use of serum and surgical material for research purposes. All samples were encoded and depersonalized.

Immunohistochemical analysis

Immunohistochemical analysis was performed on series of 4-5 μm sections from paraffin-embedded tissue. Rabbit anti-human antibodies (Dako Cytomation, Denmark, Diagnostic BioSystems, USA, ThermoScientific, USA, GeneTex, Bioworld Technology, USA) were used for staining according to

manufacturer's instructions. The presence of brown staining was considered a positive result of ER, PR, Her2/neu, Ki-67, E-cadherin, N-cadherin, FTH1, Hsp expression.

To estimate the results classic H-Score method was used:

$$\text{H-Score: } S = 1 \times N_{1+} + 2 \times N_{2+} + 3 \times N_{3+}$$

where S – «H-Score», N_{1+} , N_{2+} and N_{3+} - number of cells with low, average and high expression. End result is presented in next grade: 50-100 points – low expression, 101-200 points – average expression, 201-300 points – high expression [9].

Samples collection for RNA isolation

The 5 ml of blood was collected in a BD vacutainer (yellow top) and was centrifuged at 1500 rpm. Serum was extracted and transferred to a conical bottom tube. Around 3-5 ml serum was obtained. Serum was stored at -80°C till further use.

Frozen tissue samples also were stored at -80°C till further use.

Total RNA isolation

Total RNA was extracted from tissues/serum using "Ribo-zol" RNA Isolation Kit (Amplisens, Russia). Isolated RNA concentration was determined on a spectrophotometer "NanoDrop 2000c" Spectrophotometer (Thermo Scientific, USA). The purity of isolated RNA was controlled by using the ratio of optical absorption values at a wavelength of 260 and 280 nm. RNA was dissolved in TE buffer and stored at -20°C .

Single-stranded cDNA was synthesized from 100 ng of total RNA using TaqMan® MicroRNA Kit for reverse transcription.

Real-Time Quantitative Reverse Transcription PCR

Preparation of reverse transcription reaction mix was performed according to the manufacturers protocol. Reverse transcription was performed at a "Tertsik" ("DNA tehnologiya", Russia). After the RT-PCR reaction product was added to the mixture of reagents to perform real-time PCR with specific conditions, to manufacturer's protocol.

QRT-PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System.

Small nucleolar RNA RNU48 was used as an endogenous control for normalization of expression. Relative expression of the studied miRNAs was identified by comparative CT method. Experiment was performed in three parallels for each sample. The threshold cycle averaged in all technical and biological replicas within each sample. Fold change between the studied miRNAs expression relative to control was calculated by the formula $2^{-\Delta\Delta\text{CT}}$ [10].

Statistical methods

Statistic analysis of the obtained data was performed using the program STATISTICA 6.0. All data were expressed as the mean \pm SD of at least 3 independent experiments. The differences between the groups were analyzed using the Student's t-test and ANOVA; $P < 0.05$ was considered to indicate statistically significant results.

To determine the variation of the selected miRNA expression insamples among different groups, the data of miRNA expression obtained by qRT-PCR were analyzed by Pearson's correlation coefficient (R).

RESULTS

Expression of miRNAs was studied depending on the main clinical-pathological parameters of breast cancer patients: patient's age, menstrual status, stage of breast cancer, the presence of regional metastases in the lymph nodes, the degree of tumor differentiation, and molecular histological subtype.

General clinical characteristics of the 120 patients enrolled in the present study are shown in Table 1.

The largest number of cases was in the age range of 41-60 years. According to the results of an examination (X-ray, ultrasound, laboratory) in 27.5% of patients were diagnosed metastases in regional lymph nodes (N1-N3), distant metastases were not found.

Due to the analysis of miRNAs in the overall group of patients, it was found that most breast cancer tissue samples are characterized by a significant decrease in expression of miR-122 and -200b (in 93.2% and 83.1% respectively). When analyzing the dependence of expression of tumor miRNA-200b and -122 on age and reproductive status of patients, it was found that samples from patients younger 60 years with preserved reproductive function and estrogen-positive breast cancer, show a significant increase in miR-200b, compared to other groups of patients. For miR-122 similar correlations was not found (Table 2).

An association of miR-122 and -200b expression and breast cancer stage was established (Fig. 1, 2). The lowest levels of miR-122 and -200b ($5,2 \pm 0,35$ and $4,7 \pm 0,36$ times respectively) identified in patients with stage III breast cancer compared to normal breast tissue.

Investigation of the expression of miR-200b in accordance to differentiation degree of breast cancer demonstrated that tumors with high and moderate differentiation are characterized by higher expression of miR-200b (at $2,4 \pm 0,61$ times) than with low-grade differentiation.

The connection between expression levels of both studied miRNAs with the presence of metastatic lymph nodes was established. Thus, in patients with metastases levels of miR-122 and -200b in the tumor were lower compared to patients without metastases. Also, we found correlation of studied miRNAs expression with molecular subtype of breast cancer. The level of miR-200b in Luminal A and B subtypes was 10.4 times lower, and miR-122 in $3,1 \pm 0,13$ and $5,8 \pm 0,69$ times lower compared with the control, respectively. The lowest levels of miR-122 and -200b were observed in HER2 / neu + ($15,2 \pm 1,58$ and $18,5 \pm 2,13$ times, compared to the control) and basal subtype, which was characterized by a decrease in both studied miRNAs in more than 20 times.

It is known that one of the functions of miR-200b is the regulation of epithelial-mesenchymal transition, as well as invasive and adhesive properties of cells breast cancer. Also, it is proved that miR-200b is involved in posttranscriptional regulation of ferritin. Given the above, we analyzed the relationship of miRNA in breast cancer cells and E-cadherin and N-, CD44 and ferritin heavy chain expression (Table 3).

As can be seen from the data in the table, there is a direct correlation between the expression of miR-200b with the level of E-cadherin and inverse correlation with the levels of N-cadherin, CD44, and ferritin in tumor tissue of patients with breast cancer.

Connection of miR-122 expression with the presence of hepcidin in breast cancer cells was established. This miRNA is involved in the hepcidin regulation because it suppresses the expression of transcription factors, thereby reducing the level of this protein. As seen from the data presented in Figure 3, the hepcidin level are inverse proportional to the level of miR-122.

These data indicate that rising of hepcidin, ferritin expression and reduced adhesive properties of breast cancer cells are a consequence of reduced expression miRNA-122 and -200b.

At the next step we analyzed the features of serum miRNA-122 and -200b expression in patients with breast cancer. Due to the analysis of circulating miRNAs, we found a similar trend to lower their levels in the overall group of patients with breast cancer. As seen from the data presented in Figure 4 and Figure 5, lower levels of miR-200b and -122 in the serum of patients with breast cancer was determined in 85% and 93% of the cancer cases, respectively, compared with healthy donors.

In particular, an inverse correlation of miR-200b and miR-122 to the stage of the disease ($r = -0.38$ and $r = -0.29$, respectively), the presence of regional metastases in lymph nodes ($r = -0.46$ and $r = -0.4$, respectively) were observed.

Dependence of examined miRNAs with molecular subtype of breast cancer was determined. Higher expression of miR-200b and miR-122 was characteristic of Luminal A subtype, while the lowest levels observed miRNAs was observed in a patients with of three-negative (basal) subtype of breast cancer (fold change for miR-200b - 8, for miR-122 - 5.4 times lower compared to other subtypes).

We determined the existence of correlation of tumor and serum miR-122 and -200b (coefficient of variance is $R^2 = 0,793$). Given the above, we can conclude that serum levels of miR-122 and -200b is analogical to the levels in cancer cell and can be used as a minimally invasive markers of breast cancer progress.

DISCUSSION

MiRNAs are small noncoding RNAs that are involved in many cellular processes and are potential biomarkers for prognosis of malignant process. Levels of miRNAs in serum and plasma are mediated by physiological and pathological processes in the body. Most miRNA genes are located in fragile sites and the forming of mutant phenotype is accompanied by changes of miRNA expression at the level of whole organism [11].

Profile of miRNAs expression is individual for every tissue. If appear any violations of homeostasis, changes in expression of a number of miRNAs, depending on the organs and tissues are examined. Most studies cover the role of miR-122 and -200b only in the tumor cells of breast cancer [12, 13]. We know that the level of miR-122 in serum may depend on the functional state of the liver and hepatitis presence that was taken into account during our study. Circulating miRNAs are stable as transported in exosomes, or with complexes of high density lipoproteins, or vesicles with Ago2 (proteins Argonaut). Levels of both miRNAs in serum of healthy donors and patients with breast cancer were independent of age and reproductive status, which is an important fact when used this miRNAs as prognostic markers [14]

Individual studies indicate that breast cancer cells are characterized by a downregulation of miR-122. Data available in the literature suggest that the expression of miR-122 is much lower in cells of breast cancer, compared with normal tissue [15]. These results are consistent with studies conducted in vitro [16], that also show reduction of miR-122 in cell lines of breast cancer compared to normal epithelial cells of the breast. In addition, today we know that over-expression of miR-122 inhibits cell growth, colony formation in vitro, and carcinogenesis in vivo [17]. These results support the notion that miR-122 can function as an inhibitor of tumor growth. Increased expression of miR-122 is capable not only to reduce stem properties of tumor cells and function as a tumor

suppressor, but also increases the response of cells to chemotherapeutic agents [18]. There is evidence that circulating miRNA-122 may be a prognostic marker for metastasis at the early stages of tumor development [19].

In particular, oncosuppressive role of miR-122 is that it regulates cell proliferation through the PI3K / Akt / mTOR / p70S6K signaling pathway and therefore artificially increased expression of miR-122 may be a promising approach for the targeted treatment of breast cancer. Affecting on Bcl-W and CCNG1, miR-122 is able to reduce the expression of metalloproteinases and disintegrines - ADAM17, ADAM10, IGF1R and MADS-box transcription factor SRF [14]. Increasing of its expression can induce apoptosis and cell cycle arrest in tumor cells by reducing the expression of Bcl-W and CCNG1. To date, are known some targets of miR-122, such as, MAP3K12, Ndr3, AldoA, Bckdk, CD320, metalloproteinase ADAM10 and SRF, which play a key role in the development of various cancers, as well as Akt, MTOR and p70S6K, which are the main components of the PI3K / Akt signaling pathway. IGF1R and PI3CG also are the functional targets of miR-122 in breast cancer cells. Clinical studies have shown an inverse correlation between the levels of IGF1R and miR-122 [12]. Also it was shown that IGF1R, communicating with its growth factor, increases levels of heavy and light chains of ferritin mRNA, which reduces sensitivity to anticancer drugs [18].

Pellinen et al. proved that miR-200b, as well as miR-122, is oncosuppressive. During breast cancer decreasing of miR-200b expression correlates with poor prognosis [20]. Increased miR-200b level leads to inhibition of proliferation and invasion of breast cancer cells in vitro, while reducing of this miRNA is characteristic of tumor cells that form metastases [21]. Reduced expression of miR -200b also is associated with reduced synthesis of E-cadherin, which can stimulate epithelial-mesenchymal transition [22]. In addition, expression of miR-200b is associated with breast cancer drug resistance through a direct effect on the expression of genes responsible for metabolism of xenobiotics, as well as hormone therapy, through the regulation of the expression of hormone receptors [23].

Our research has shown that levels of miR-122 and -200b in serum allow to distinguish patients with HER2 + and basal molecular subtype of breast cancer from healthy donors, that makes them valuable prognostic markers in BC. In addition, expression of two miRNAs correlates with the presence of metastases in lymph nodes.

Established dependence of hepcidin and miR-122 expression in tumor tissue also consistent with the literature. As we know, miR-122 is involved in the regulation of system iron homeostasis [24], by influencing the hepcidin expression. This miRNA is capable to inhibit hepcidin transcrip-

tion by regulation of its transcription factors, namely human hemochromatosis protein (HFE), hemojuvelin (HJV), Tfr2, bone morphogenetic protein (BMP) -6 and Smad4 protein, that directly regulate Hamp transcription [24].

MiR-200b also is involved in the regulation of iron metabolism - it regulates the synthesis of heavy (Fth1) and light (Ftl) ferritin chains, which converts excess redox iron into inactive form, preventing chronic redox stress in the cell [25].

The above is consistent with our previous studies on the relationship of hepcidin and ferritin levels with breast cancer [26]. In particular, the levels of circulating miR-122 and -200b are inversely proportional to hepcidin and ferritin levels in the blood of patients with breast cancer and correlate with major clinical - pathological parameters of BC.

Established correlation of miR-200b levels with molecular subtype of breast cancer in patients is consistent with Humphries et al, study, which shown lower levels of expression of the whole family of miR-200 in metastatic basal BC compared to other subtypes of breast cancer. Artificial restoration of normal expression of miR-200b reduces migration and metastasis of tumor cells. It was also shown that protein kinase α (PKC α) is the target of the miRNA and its inhibition also reduces the metastatic potential of the cells of the basal subtype [27].

Found correlation of expression of miR-200b with cell differentiation grade coincides with measured levels of E- and N-cadherin, and with literature data. In particular, Yu J et al proved feedback of miR-200b expression and increased levels of E-cadherin and CD44 in prostate cancer cells [28].

Although there is evidence on the relationship of expression of the marker CD44 in stem cells and EMT, we first show the correlation of miR-200b and CD44. It is associated with miR-200b involvement in metastasis - epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) [29].

EMT / MET are mediated by ZEB1 and ZEB2 transcription factors, responsible for the reduction of miR-200b, which leads to loss of E-cadherin expression and subsequent formation of EMF phenotype. There is also a feedback - mRNA ZEB1 and ZEB2 are targets of miR-200b, which stimulates mesenchymal-epithelial transition [30].

Target for miR-200b is also a RAB family. RAB-GTPases regulate membrane traffic, including the formation of vesicles and their movement along actin and tubulin. In addition, RAB family also is associated with invasion and metastasis [20, 32].

This universal oncosuppressive role of miR-122 and -200b in cancer makes them universal markers of BC, regardless

of intratumor heterogeneity as these miRNAs are involved in the regulation of virtually all important cancer triggers. Whether what molecular violations are specific to a particular clone of breast cancer cells, the levels of these miRNAs give an idea of the overall degree of malignancy, due violation of their regulation occurs at lower adhesion and strengthening of migratory properties of tumor cell, changes in metabolism, energy balance not only in the area of the tumor, but also at the level of the organism (as hepcidin affects glucose metabolism), etc.

We established the connection of changes in the levels of miR-122 and 200b in serum and tumor tissue. Given the fact that obtaining serum samples is less invasive and traumatic than the samples of tumor tissue, we analyzed the correlation between the expression of miRNAs in serum and tumor. Our results showed a high degree of correlation of expression profile of circulating and tumor miR-122 and -200b.

CONCLUSION

Heterogeneity of breast tumors is mediated by multiple signaling pathways and requires integrated approaches for diagnosis and prognosis of cancer. In this aspect, the expression of miRNAs can be not only an indicator of the complexity of the molecular profile of breast cancer, but also easy to use prognostic marker. The potential use of miR-122 and -200b as the panel is more statistically significant as compared with expression of only one of them. Established data are the basis for the development of epigenetic prognostic criteria to improve existing methods of breast cancer prognosis.

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Table 1: General clinical-pathological characteristic of breast cancer patients

	Number of cases	
	N	%
Total patient's number	120	100
Age (years)		
Average	55,4±7,3	
Median	23 – 89	
Menstrual function		
Preserved	33	27,5
Menopause	87	72,5
TNM stage		
I	26	21,7
II	58	48,3
III	36	30
Lymph nodes metastases (N)		
No	85	70,8
N ₁₋₃	33	27,5
N _x	2	1,7
Histological type		
Invasive ductal carcinoma	82	68,3
invasive medullary carcinoma	38	31,7
Differentiation grade		
G ₁ (well-differentiated)	33	27,5
G ₂ (intermediately- differentiated)	60	50
G ₃ (poorly-differentiated)	27	22,5
Molecular subtype		
Luminal A	56	46,7
Luminal B	27	22,5
Basal	27	22,5
Her2/neu+	10	8,3

Table 2: Association of miR-122 and -200b expression with ER status of human BC

Age	Number of subjects	Fold change (mean±SD)			
		ER-		ER+	
		miR-200b	miR -122	miR -200b	miR -122
> 60 (non menopausal)	33	-5,36±1,56*	-2,56±1,48	- 3,23±1,48*	-3,35±1,89
>60 (menopause)	87	-6,59±1,94*	-4,76±1,83	-5,93±2,32*	-3,96±1

*p≤0.05

Table 3: Level of miR-200b correlates with E-cadh, N-cadh, CD44 and ferritin expression in human BC cells

	E-cadh (mean±SD, H score)	N-cadh (mean±SD, H score)	CD44 (mean±SD, H score)	ferritin (mean±SD, H score)
miR-200b expression <10 times lower compared to normal tissue	280±3,0	5±0,9	30±1,2	95±6,93
miR-200b expression ≥10 times lower compared to normal tissue	20±1,1	260±2,1	190± 3,1	256±8,12
Correlation	0,36	-0,4	-0,28	-0,37

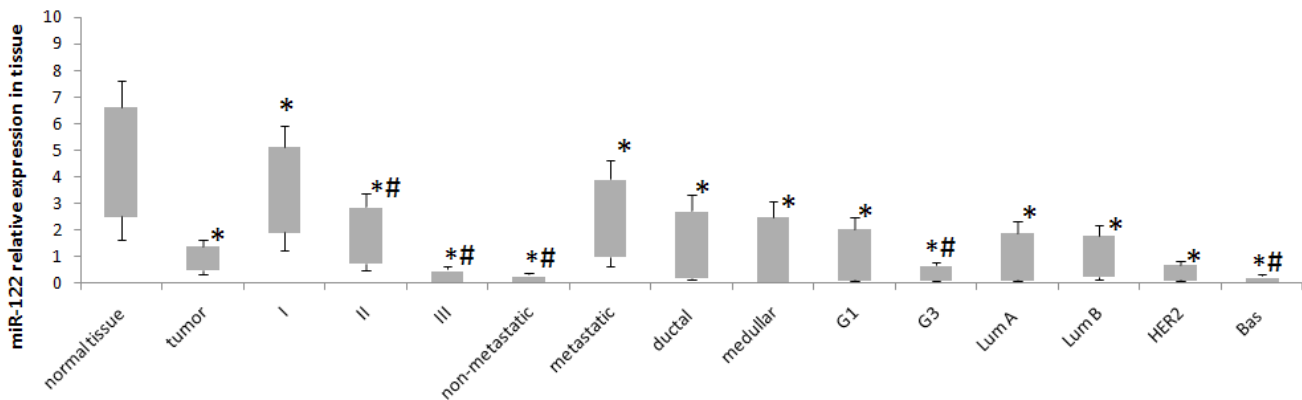


Figure 1: Expression of miR-122 in tumor tissue is associated with major clinical-pathological characteristics of BC patients.

*p≤0.05 compared to normal tissue

#p≤0.05 compared to similar characteristics

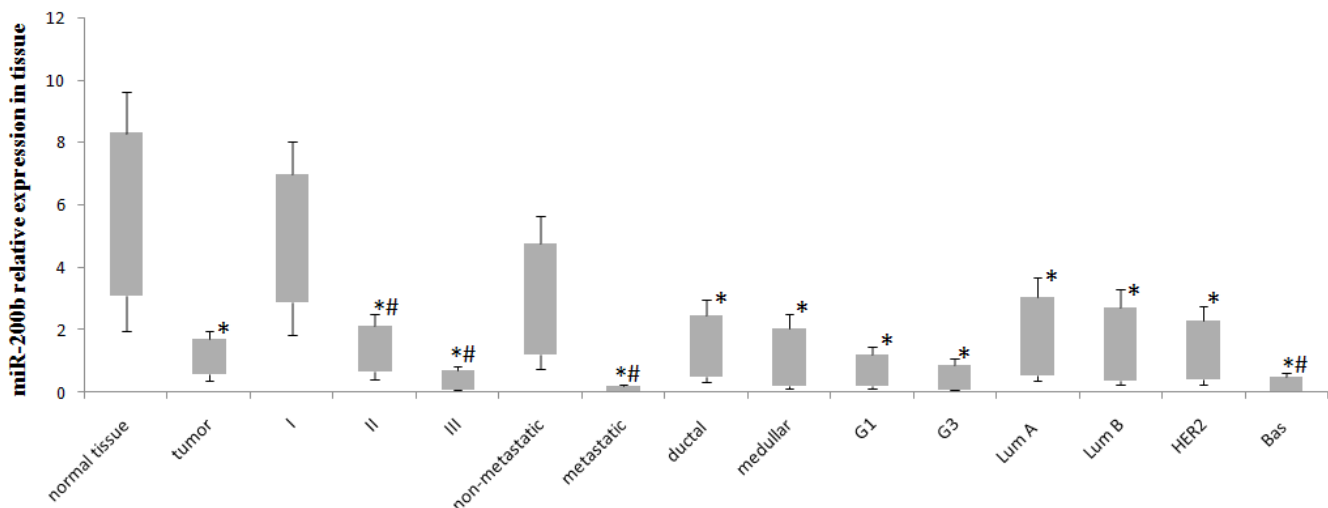


Figure 2: Expression of miR-200b in tumor tissue is associated with major clinical-pathological characteristics of BC patients.

*p≤0.05 compared to normal tissue.

#p≤0.05 compared to similar characteristics

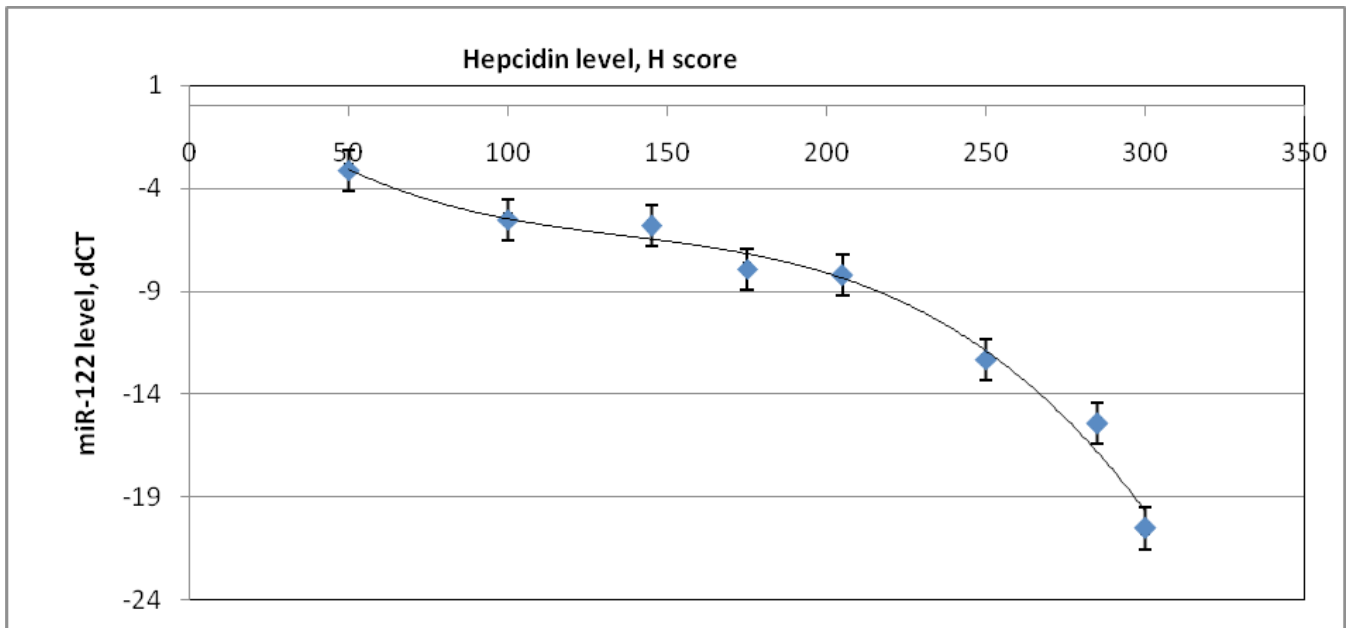


Figure 3: Level of miR-122 is inversely proportional to hepcidin expression in BC cells.

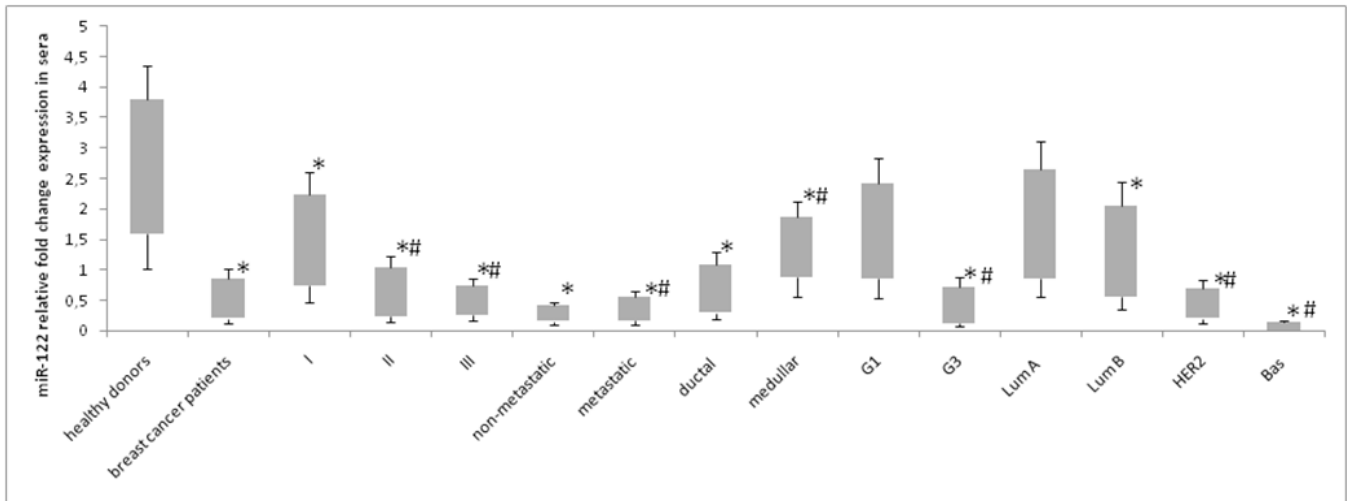


Figure 4: Expression of miR-122 in serum of BC patients is associated with major clinical-pathological characteristics of BC patients.

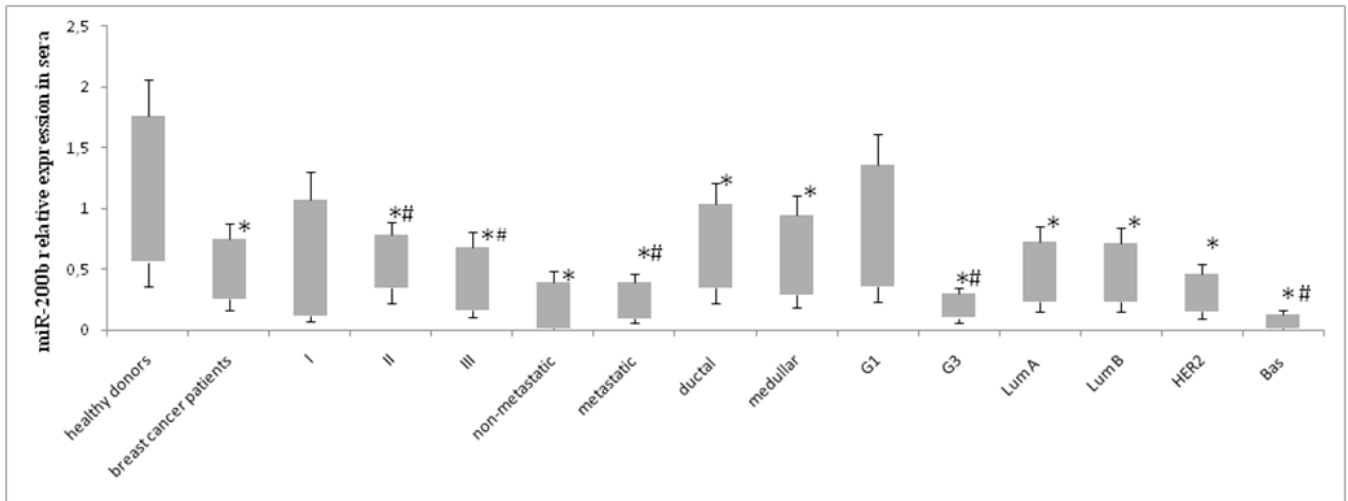


Figure 5: Expression of miR-122 in serum of BC patients is associated with major clinical-pathological characteristics of BC patients.

* $p \leq 0.05$ compared to healthy donors

$p \leq 0.05$ compared to similar characteristics