miRNA 200 Family Expression is a Strong Predictor for Recurrence Following Liver Resection for Breast Cancer Liver Metastases

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ABSTRACT

Although only a small percent of breast cancer patients are diagnosed in metastatic stages, a significantly higher percent will develop disseminated recurrences at a certain point of their evolution, this event being responsible for a high number of cancer related deaths. In recent years a particular attention was paid for identifying the factors which might be responsible for the development of metastatic disease. The current study included 27 patients submitted to surgery for breast cancer liver metastases in “Dan Setlacec” Center of Gastrointestinal Diseases and Liver Transplantation Fundeni Clinical Institute, Bucharest Romania. In all cases miRNA expression was studied in both tumoral and non-tumoral tissues. miRNA species which were proved to be supra-expressed in the tumoral areas included mainly the miRNA 200 family (miR-200c and miR-141). These results demonstrate that alterations in miRNA-200 family play a key role in development of breast cancer liver metastatic process. In the meantime miRNA family seems to be a strong predictor for recurrence following liver resection for breast cancer liver metastases. Therefore, these molecules might become an useful signature in identifying high risk breast cancer patients; moreover this analysis seem to provide a better selection of patients who could benefit most from hepatic resection for breast cancer liver metastases. In this way, a significant improvement of overall survival after liver resection is expected. In the meantime, miRNA analysis could also indicate which patients are less probably to benefit from surgery and could straighten them to other personalized therapeutic strategies.

Key words: breast cancer liver metastases, miRNA 200 family, liver resection

INTRODUCTION

Although only up to 10% patients with breast cancer present with metastatic disease from the initial time of diagnostic, a significantly higher number of cases with develop metastatic disease at a certain point of their evolution; this

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eventuality seems to be highly influenced by the tumor type, as well as by the lymph node status. Due to the fact that most cases presenting metastatic lesions will have a high clinical burden of metastatic disease (disseminated lesions involving different viscera) and they will become candidates only for palliative treatment (1-3).

Moreover, it seems that although patients diagnosed in similar stages of the disease, with similar histopathological subtypes receive standard treatment, their evolution is not quite similar; therefore, it has been suggested that there are other factors which might significantly influence the prognosis and the long term outcomes of such cases (4-6). These facts enabled the researchers to consider that the current classification of breast cancer is far from being perfect, an important number of cases receiving an ineffective or incomplete treatment. Therefore, these patients will experience recurrence in spite of an initially presumed good long term prognosis (7). This is why in the last decades it has been widely accepted that the concept that "one medicine fits for all patients with the same disease" is no longer valid and the concept of personalized cancer therapy was proposed (8).

In consequence, attention was focused on determining which the specific prognostic factors that might influence the risk of development of distant metastases are; once these factors are revealed, patients at high risk of developing systemic recurrences might receive a more specific treatment which might impede the apparition of disseminated metastatic disease (1-3,9,10). A certain role in developing distant metastases irrespective of the origin of the primary tumor seems to be played by the presence of deregulated genes which seem to represent a prognostic factor in order to predict the occurrence of distant lesions (7,11-14). In the last decade a particular attention was paid for investigating the role of miRNA in breast cancer progression.

MATERIAL AND METHOD

Data of all patients submitted to hepatic resections for breast cancer liver metastases in "Dan Setlăceș" Center of Gastrointestinal Diseases and Liver Transplantation Fundeni Clinical Institute, Bucharest Romania were retrospectively reviewed after obtaining the approval of the Ethical Committee (no.30504/17.10.2017). In the meantime, a written consent from all patients had been obtained before beginning the study. For each patient a numeric code was provided in order to maintain the confidentiality and anonymity of

the patients. In all cases the specimens were obtained from both breast cancer liver metastases and normal hepatic tissues and were harvested each in 5 ml RNA Later (Sigma) solution in order to prevent RNA destroy; after this all specimens were placed at -85°C while for isolation of the RNA molecules the frozen tissues were stocked in RNAlater (Qiagen). The concentration of total RNA was determined by the spectrophotometric method using Nano-Drop 1000 (Thermo Scientific). Checking out the samples was performed by capillary electrophoresis in acrylamide polymer. All used samples had a miRNA concentration higher of 200ng/ul and a percentage concentration of miRNA higher than 25% of the entire quantity of isolated RNA. In order to select only the conform RNA samples, miRNA Microarray System with miRNA Complete Labeling and Hyb Kit (Agilent Technologies) and G3 Human miRNA Microarray Kit, Release 21, 8×60K (Agilent Technologies) protocols were used. Data was generated using Feature Extraction 12.0.3.1 soft. All quality reports were classified as "excellent" from technical point of view (fig. 1).

RESULTS

Based on the reports of quality and integrity of the RNA samples, 27 cases were considered eligible for the current study. The total RNA samples were analyzed based on the spectrophotometric studies while the miRNA samples were analyzed through capillary electrophoresis in acrylamide polymer. Data obtained are shown into *table 1*.

Data regarding miRNA expression obtained through microarray for the 27 patients with breast cancer liver metastases were analyzed using Gene Spring Software 14.8. miRNA expressions for tumoral and non-tumoral hepatic tissues are presented in *fig. 2*.

The next step was realizing a Heatmap report using data regarding miRNA levels in tumoral and non-tumoral liver tissues for both under-expressed and supra-expressed miRNA molecules. The under-expressed molecules included miR-122-5p, miR-122-3p, miR-3962, miR-5195, miR-486-5p, miR-6814, miR-885-5p while the supra-expressed ones included miR-141-3p, miR-183-5p, miR-196a, miR-4449, miR96-5p (*fig. 3 a,b*).

Reported results were next corrected using Benjamini-Hochberg correction; therefore two species of supra-expressed miRNA molecules (miR-200c-3p și miR-141-3p) and under-expressed molecules (miR-122-5p și miR-122-3p) were obtained. Comparative analysis of miRNA was realised by measuring their expression in metastatic tissue as well as in the resection margin's

Figure 1 a,b - The qualitative report of miRNA microarray

Table 1 - Data regarding the RNA samples isolated from the 27 cases

No. of case	Patient's code	Type of hepatic tissue	Concentration ng/ul
1	GDA-5	tumoral	847.48
		normal	1246.01
2	FR-4	tumoral	1985.97
		normal	625.51
3	SM-23	tumoral	1537.85
		normal	955.03
4	NM-36	tumoral	2015.50
		normal	3500.89
5	NM-43	tumoral	1360.49
		normal	101.79
6	MM-8	tumoral	581.61
		normal	1856.53
7	EC-32	tumoral	1548.86
		normal	1610.30
8	MA-15	tumoral	785.07
		normal	299.74
9	AF-2	tumoral	1067.51
		normal	426.02
10	ND-41	tumoral	409.46
		normal	1199.11
11	AA-53	tumoral	161.62
		normal	391.17
12	IL-54	tumoral	600.89
		normal	1422.91
13	BD-56	tumoral	436.13
		normal	565.09
14	CDE-57	tumoral	857.37
		normal	572.13
15	LAG-58	tumoral	653.88
		normal	400.43
16	TA-59	tumoral	1177.19
		normal	503.77
17	SI-60	tumoral	409.20
		normal	1137.89
18	MC-61	tumoral	454.76
		normal	2183.25
19	VA-62	tumoral	308.73
		normal	124.24
20	IM-63	tumoral	360.60
		normal	955.67
21	BD-48	tumoral	233.61
		normal	395.43
22	ID-64	tumoral	1145.22
		normal	1231.05
23	SA-65	tumoral	360.04
		normal	743.63
24	VAM-25	tumoral	895.22
		normal	1015.13
25	HS-52	tumoral	310.77
		normal	105.94
26	MC-35	tumoral	463.60
		normal	450.71
27	PM-66	tumoral	1184.94
		normal	130.38

Figure 2 - miRNA levels obtained through microarray. a) BoxWhiskerReport of under-expression
b) BoxWhiskerReport of supra-expression

Figure 3 - a) Heatmap report – under-expressed miRNA. b) Heatmap report – supra-expressed miRNA

tissues. Data are shown in *table 2*. miRNA species which were proved to be under-expressed in metastatic hepatic areas (miR-122-5p and miR-122-5p) are in fact specific molecules for normal hepatic tissues while supra-expressed molecules (miR-200c and miR-141) are especially present in epithelial tissues.

DISCUSSIONS

The anarchic growth and spread of cells from a

primary tumor is in fact the most common cause of death in cancer patients; at cellular level, these processes are characterized by the contact loss with the neighboring cells, increasing in this way their invasively character. This process is in fact represented by the epithelial to mesenchymal transition, a process in which epithelial cells experience a modification of their morphology, becoming more likely to mesenchymal cells. The resulting cells have a higher capacity of spread and developing distant tumoral lesions (15).

Table 2 - The main miRNA molecules which proved to be under- or supra-expressed in breast cancer liver metastases

ID	FC	AvgExpr	t	adj.P.Val
hsa-miR-200c-3p	17.54	29.6	4.2	8.8E-03
hsa-miR-141-3p	21.68	27.9	4.2	8.8E-03
hsa-miR-122-5p	0.06	1,694.0	-3.9	1.9E-02
hsa-miR-122-3p	0.05	24.8	-3.5	3.8E-02

Several studies have demonstrated that a central role in epithelial to mesenchymal transition is played by miRNA molecules, which seem to act as oncogenes or tumor suppressor (16).

microRNA (miRNA) are represented by short non-coding single stranded molecules of ARN composed by approximately 22 nucleotides which have the property of binding complementary sequences on target messenger RNA transcripts (17). This fact results in repression of the translational process and gene silencing; therefore miRNA regulate various biological functions such as cellular growth, differentiation and apoptosis, playing a central role in tumor progression (17,18). Due to the fact that a single miRNA might regulate more different genes, it can become a very attractive therapeutic agent which might provide a promising way to control tumoral development. Moreover, it seems that in tumoral tissues miRNA can be overexpressed or under expressed when compared with normal tissues, this fact being able to explain the role of oncogene or tumor suppression gene of miRNA (18,19).

According to McGuire's study, among miRNA molecules identified in breast cancer patients, there are few particles which play a key role in dissemination as well as in the metastatic cascade: while miR-199a, miR-214, miR-200a, miR-200b, miR-200c, miR-141 and miR-429 play a crucial step in tumor cell intravasation, miR-31 plays a central role in dissemination while miR-10b, miR-34-a, miR-155, miR-200a, miR-200b, miR-200c, miR-141 as well as miR-429 represent basic molecules in order to induce proliferation in the metastatic cellular clusters (20). This fact was also sustained by our findings, a higher expression of miR-200c and miR-141 being observed at the level of the liver metastases.

An interesting correlation was established between miR-200, E-cadherin and the risk of developing distant metastases. It has been widely demonstrated that the invasiveness of a tumoral cell is inversely correlated with E-cadherin expression, its' overexpression suppressing invasion (21-23). However, E-cadherin expression is usually down-regulated by miRNA-9, miRNA-92 or miR-200, the main mechanism which is responsible for this process consisting of direct suppression or inhibition of transcribing factors responsible for E-cadherin's regulation. Therefore increased levels of miR200, miRNA-9 or miRNA-92 are strongly correlated with decreased E-cadherin's activity, this fact inducing the apparition of distant metastases; however this correlation could not be demonstrated for cancers that had metastasized to the brain (24). When it comes to

the live metastases, it seems that a higher level of miR200 could play a certain role, as demonstrated by our study too.

The miR-200 family is in fact composed by 5 molecules (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) which has been related to breast cancer progression; initially this family inhibits the epithelial to mesenchymal transition, one of the initial steps in metastatic spread of the tumoral cells. Later on during this process, miR-200 influences the ability of colonization and growth in distant metastatic sites by influencing the reverse process, mesenchymal to epithelial transition (25-28). An interesting study which came to demonstrate the role of miR-200 family in the metastatic process in breast cancer patients was published by Karina Gravaard in 2012. The authors demonstrated that miR-200 as well as miR-9 molecules report different expressions at different stages of progression of the malignant process; while in the initial stages of the disease the miR-200 family has been found to be down regulated, in metastatic stages they will be found in increased amounts (18).

A study conducted on the theme of differential expression of miRNA in breast cancer patients and a further correlation with lymph node status was conducted by Rask and published in 2014. The study included 43 fresh frozen samples of invasive ductal carcinoma selected from breast carcinoma biobank in Herlev Hospital, Denmark between 2005 and 2007 (7). In order to obtain a homogenous cohort, the authors included in the study only hormone positive tumors (defined as estrogen or progesterone receptor expression of at least 10%) with negative status for HER2 overexpression; in the meantime all tumors measured between 1-3cm. when it came to the lymph node status, 20 patients presented at least one lymph node metastasis while the remaining 23 cases presented no lymph node metastasis. When analyzing the miRNA distribution among the two groups, the authors demonstrated that patients with positive lymph nodes reported 17miRNAs with a significant difference in terms of expression between the two groups (positive and negative lymph node groups), 8miRNAs being up-regulated and 9miRNAs being down-regulated in the positive lymph node group when compared to the negative lymph node group ($p < 0.01$). Among these molecules, up-regulation of miR-21 was significantly associated with breast cancer lymph node metastases why down-regulation of miR-355 was associated with a higher risk of recurrence when compared to patients presenting normal levels of miR-355. Moreover, the authors demonstrated that miR-486-5p, miR-139-5p,

miR-223 and miR-455-3p were all found to be down-regulated in cases presenting lymph node metastases. However, when investigating the other parameters (such as age at diagnostic or tumor size) no significant correlation could be established between them and miRNA expression patterns (7).

CONCLUSIONS

Although understanding metastatic breast cancer has significantly advanced during the last decades, development of disseminated tumoral recurrences remains the major cause of death in such patients. Over the last decade significant progress has been made in understanding the mechanisms of dissemination once microRNA molecules have been investigated. Also defined as “small RNAs which play big roles”, these molecules seem to act as oncogenes, tumor suppressors or metastasis regulators which influence migration, invasion colonization or epithelial – mesenchymal transition (29). miRNA analysis in breast cancer patients play a crucial role in order to identify the subset of patients in whom development of distant metastases is to be expected. In consequence, these patients might benefit from a personalized therapeutic strategy. In conclusion, miRNA analysis represent a crucial step in order to identify patients who could benefit most from hepatic resection for breast cancer liver metastases, significant improvement of the overall survival after surgery being expected; therefore, patients presenting supra-expression of miRNA 200 family at the level of the hepatic lesions seem to have the best long term outcomes after liver resection for breast cancer liver metastases. Moreover, the same analysis might help the surgeon to exclude from surgical treatment cases in which resection does not seem to increase survival and propose this particular category of patients for other personalized therapeutic options.

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