RESEARCH COMMUNICATION

Differential Distribution of miR-20a and miR-20b may Underly Metastatic Heterogeneity of Breast Cancers

Jian-Yi Li[&], Yang Zhang, Wen-Hai Zhang[&*], Shi Jia, Ye Kang, Xiao-Yu Zhu

Abstract

Background: The discovery that microRNA (miRNA) regulates metastasis provide a principal molecular basis for tumor heterogeneity. A characteristic of solid tumors is their heterogenous distribution of blood vessels, with significant hypoxia occurring in regions (centers of tumor) of low blood flow. It is necessary to discover the mechanism of breast cancer metastasis in relation to the fact that there is a differential distribution of crucial microRNA in tumors from centers to edges. Methods: Breast tissues from 48 patients (32 patients with breast cancer) were classified into the high invasive and metastatic group (HIMG), low invasive and metastatic group (LIMG), and normal group. Samples were collected from both the centers and edges of all tumors. The first six specimens were detected by microRNA array, and the second ten specimens were detected by real-time qRT-PCR and Western blot analyses. Correlation analysis was performed between the miRNAs and target proteins. Results: The relative content of miR-20a and miR-20b was lower in the center of the tumor than at the edge in the LIMG, lower at the edge of the tumor than in the center in the HIMG, and lower in breast cancer tissues than in normal tissues. VEGF-A and HIF-1alpha mRNA levels were higher in the HIMG than in the LIMG, and levels were higher in both groups than in the normal group; there was no difference in mRNA levels between the edge and center of the tumor. VEGF-A and HIF-1alpha protein levels were higher in the HIMG than in the LIMG, and protein levels in both groups were higher than in the normal group; there was a significant difference in protein expression between the edge and center of the tumor. Correlation analysis showed that the key miRNAs (miR-20a and miR-20b) negatively correlated with the target proteins (VEGF-A and HIF-1alpha). Conclusions: Our data suggest that miR-20a and miR-20b are differentially distributed in breast cancer, while VEGF-A and HIF-1alpha mRNA had coincident distributions, and VEGF-A and HIF-1alpha proteins had uneven and opposing distributions to the miRNAs. It appears that one of the most important facets underlying metastatic heterogeneity is the differential distribution of miR-20a and miR-20b and their regulation of target proteins.

Keywords: Heterogeneity - breast cancer - metastasis - miR-20a - miR-20b

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Introduction

Metastasis is the main reason that patients with breast cancer experience treatment failure and death (Desantis et al., 2011). However, the mechanism underlying tumor metastasis is still poorly understood. The discovery of microRNA (miRNA) regulation of metastasis is considered to be part of the principal molecular basis of tumor heterogeneity (Voorhoeve, 2010). Heimann's study in 1998 and long-term clinical follow-up found that the probability of breast cancer metastasis increased with tumor size (Heimann and Hellman, 1998). Interestingly, the solid tumor is not homogeneous; rather the characteristic of solid tumors is their heterogenous distribution of blood vessels, with significant hypoxia occurring in the center regions of low blood flow (Balsat et al., 2011; Osinsky et al., 2011). It was necessary to discover the mechanism of breast cancer metastasis in a direction that there has been differential distribution of crucial microRNA in tumors from center to edge.

Materials and Methods

Patients and Groups

Forty-eight human breast tumor tissues were obtained by surgical resection from patients treated at Shengjing Hospital of China Medical University (SJHCMU) from 2009 to 2011. Inclusion criteria included invasive ductal carcinoma, tumor diameter between 2 and 3 cm, no family history of neo-adjuvant therapy and radiotherapy, no family history of other cancers, and no accessory breast cancer. Of those patients, 16 met the requirements and entered the High Invasive and Metastatic Group (HIMG). The patient characteristics in this group were: lymph node metastases, histological grade III, Her2-positive, vascular cancer embolus-positive, estrogen and progesterone receptor-negative, p53-positive, and Ki67 index greater than or equal to 14%. Sixteen patients met the requirements for the Low Invasive and Metastatic Group (LIMG) which included: no lymph node metastases or micro-metastases, histological grade I, Her2-

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Table 1. Grouping Criteria

Grouped Criteria	High invasive and metastatic group HIMG	Low invasive and metastatic group LIMG
Lymph Nodes Metastasis by HE	Yes	No
Micro-Metastasis by CK-22	Unnecessary	No
Histological Grading	III	I
Tumor Embolus	Positive	Negative
Her2 receptor Status	Positive	Negative
ER & PR	Negative	Positive
P53	Positive	Negative
Ki67	<14%	<14%

Illustration: All indicators of immunohistochemical staining need to meet verification of two pathological diagnosis centers

Table 2. Patients Parameters

Parameters	HIMG	LIMG	Normal Group
Age(years)			
Median	52.5	55.5	50
Range	(40~63)	(46~67)	(33~68)
Menopause			
Yes	7	8	5
No	9	8	11
Quadrant			
Areolar	2	1	2
Outer upper	8	7	10
Outer lower	4	6	2
Inner lower	2	1	1
Inner upper	0	1	1
Operation			
Mastectomy	14	12	0
Tumorectomy	2	4	16
Diameter	2.53±0.30	2.48±0.29	

Illustration: There was no significance difference among the three groups (HIMG, LIMG and Normal Group) in all of the parameters

negative, vascular cancer embolus-negative, estrogen and progesterone receptor-positive, p53-negative, and Ki67 index less than 14% (Table 1). We randomly chose 16 patients with benign tumors and normal breast tissues for the control group (Table 2). All patients gave informed consent. Each group of 16 specimens was randomly divided into two sections: the first section consisted of 6 cases that were used for microRNA array screenings, and the second section consisted of 10 cases that were used for validation experiments by realtime RT-qPCR and Western blot analyses.

Tumor samples

The largest section of the tumor, which was parallel to the chest wall and more than 3 mm thick, was obtained by open surgery. The center and edge of the tumor were determined by the naked eye, and the weight of each specimen was more than 30 mg (Figure 1). The same quantity of normal breast tissue was obtained from patients in the control group. All samples were stored in the freezer (<80 °C) after quick-freezing in liquid nitrogen.

MicroRNA Array

For each group of six samples that were randomly selected, the center and edge of the tumor were mixed

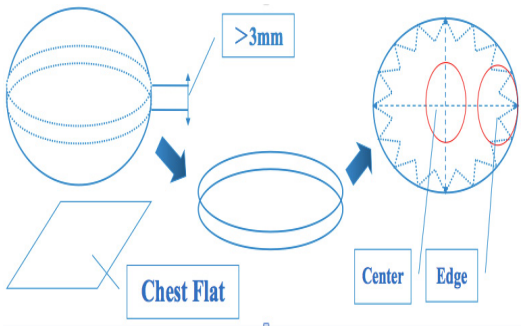

Figure 1. Diagram of Tumor Partition

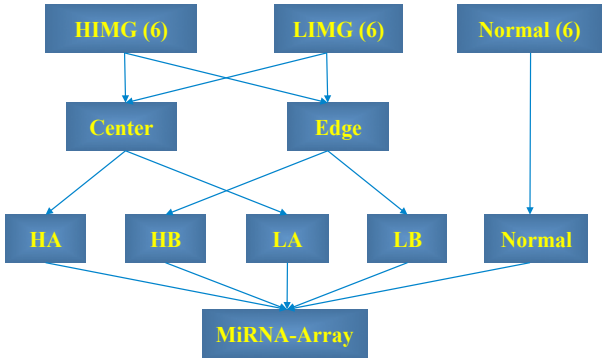

Figure 2. Diagram of Specimen Mixed

together with a mixed sample from the control group; there were five mixed samples (the center part of HIMG-HA, the edge part of HIMG-HB, the center part of LIMG-LA, the edge part of LIMG-LB, Normal) that were screened by microRNA Arrays (Figure 2).

The 6th generation of miRCURYTM LNA Array (v.16.0) (Exiqon; Vedbaek, Denmari) contains more than 1891 capture probes, covering all human, mouse and rat microRNAs annotated in miRBase 16.0, as well as all viral microRNAs related to these species. In addition, this array contains capture probes for 66 new miRPlus™ human microRNAs. Total RNA was isolated using TRIzol (Invitrogen; Carlsbad, CA, USA) and the miRNeasy Mini Kit (QIAGEN, Spoorstraat, Netherlands) according to the manufacturer's instructions, which efficiently covered all RNA species, including miRNAs. RNA quality and quantity were measured using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, USA) and RNA integrity was determined by gel electrophoresis.

After RNA isolation from the samples, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon) was used according to the manufacturer's guidelines for miRNA labeling. One microgram of each sample was 3'-end-labeled with Hy3TM fluorescent label, using T4 RNA ligase according to the following procedure: RNA in 2.0 µl of water was combined with 1.0 µl CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37 °C, and was terminated by incubation for 5 min at 95 °C. Then, 3.0 µl of labeling buffer, 1.5 µl of fluorescent label (Hy3TM), 2.0 µl of DMSO, and 2.0 µl of labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16 °C, and terminated by incubation for 15 min at 65 °C. After termination of the labeling procedure, the Hy3TM-labeled samples were

Table 3. Primers

Universal reverse transcription primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(TT…TT)24N(A, G, C)	
U6	F: CTCGCTTCGGCAGCACA	R: AACGCTTCACGAATTTGCGT
miR-20a	F: AAGTGCTTATAGTGCAGGTA	R: TGTCAACGATACGCTACG
miR-20b	F: GCTCATAGTGCAGGTAGAA	R: TGTCAACGATACGCTACG
VEGFA	F: CAACTTCTGGGCTGTTCT	R: TCTCCTCTTCCTTCTCTTCT
HIF-1	F: AGCACAGTTACAGTATTCCA	R: AATGTCTTCCATACGGTCTT
GAPDH	F: GGTGAAGGTCGGAGTCAACG	R: CCATGTAGTTGAGGTCAATGAAG

Table 4. MicroRNA-Array

ID	Name	HA	HB	LA	LB	normal
145845	hsa-miR-20a	1.75371802	0.25285481	0.57437408	2.13344316	4.82939363
42640	hsa-miR-20b	0.196312	0.065253	0.092784	0.24547	0.641316

Illustration: HA means the center of HIMG, HB the edge of HIMG, LA the center of LIMG and LB the edge of LIMG

hybridized on the miRCURYTM LNA Array (v.16.0) (Exiqon) according to the instructions in the array manual. The total 25 μl mixture of Hy3TM-labeled samples and 25 μl hybridization buffer were denatured for 2 min at 95°C, incubated on ice for 2 min, and then hybridized to the microarray for 16–20 h at 56 °C in the 12-Bay Hybridization System (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixed action and constant incubation temperature to improve hybridization uniformity and enhance signals. Following hybridization, the slides were washed several times with wash buffer from the Exiqon kit, and finally dried by centrifugation for 5 min at 400 rpm. The slides were then scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments; Foster City, CA). Scanned images were imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥ 50 in all samples were chosen for calculating the normalization factor. Expressed data were normalized using Median normalization. After normalization, differentially expressed miRNAs were identified through Fold Change filtering. Hierarchical clustering was performed using Multiple Experiment Viewer (MEV) software (v4.6, TIGR).

Realtime qRT-PCR

Small RNA and total RNA from the breast tissue was extracted using the mirVana™ miRNA Isolation Kit (AM1560, ABI, USA). Reverse transcription was performed with the PrimeScript® RT reagent kit (DRR037A, Takara, Japan) in a final volume of 10 μl containing 200 ng RNA and other elements according to the protocol. Poly A tail was added to the small RNA by poly A polymerase (NEB, M0276) before reverse transcription using primers outlined in Table 3 (Table 3).

Real time quantitative PCR was performed on aRoche Light Cycler 2.0 with SYBR® Premix Ex Taq™ (Takara, DRR041A). For each sample, real time PCR was performed in a final volume of 10 μl containing PCR master mix, 50 ng genomic DNA or 5 ng cDNA, and primers (250 nM). For the negative controls, the template was replaced with purified non reverse-transcribed RNA. Each experiment was done in triplicate. Average GAPDH Ct values were subtracted from each average Ct value of interest to give ΔCt.

Western blot analysis

Protein extracts, SDS-PAGE, electrotransfer and immunoblotting were performed according to standard procedures. VEGF-A and hypoxia-inducible factor-1alpha (HIF-1alpha) expression were detected by antibody sc-6836 (Santa Cruz, USA), raised against the C-terminal of VEGF-A and HIF-1alpha. A GAPDH antibody was used as an internal control (KC-5G4, Kangchen Biotech, China). Densitometric analysis was done using Quantity One (version 4.5, Bio-Rad, USA).

Statistical Analysis

Key miRNAs verified by Realtime qRT-PCR were analyzed using the public miRBase database (www.microrna.org), and the target protein and pathway was analyzed using the public KEGG database (www.genome.jp). A normality test was performed on all data. Normally distributed data were compared by the t test, a log-transformation was performed on other data to enable normal distribution, and abnormally distributed data were analyzed using the Mann-Whitney U test. Multiple groups were compared by ANOVA analysis, the Student-Newman-Keuls (SNK) test was used to analyze data between the groups, and the Person test was used for correlation analysis. P values less than 0.05 were defined as statistically significant. Statistical analysis was performed using SPSS software (version 17.0).

Results

Differential Distribution of MicroRNA in Tumors (miR-20a and miR-20b)

MicroRNA array screening results (from 18 samples, 3 groups of six specimens) showed an order of expression of miR-20a and miR-20b from low to high that was ranked as HB<LA<HA<LB<Normal (Table 4). According to the testing standard, which states that the value of Fold Change must be more than 2 or less than 0.5, the four combinations above were significant, including HB<HA<Normal, LA<LB<Normal, HB<LB,Normal, and LA<HA<Normal (Figure 3). The relative content of miR-20a extracted from breast tissue in normal tissues was 0.00±2.46, in LA was -6.45±1.53, in LB was -2.31±2.38, in HA was -3.90±2.43, and in HB was -10.05±2.10. The relative content of miR-20b extracted from breast tissue in normal tissues was 0.00±1.92, in LA was -9.03±2.26,

Table 5. Target Proteins & Regulatory MiRNAs

MicroRNA	Targets	Regulatory Sequences	mirSVR score	PhastCons score
miR-20a	VEGF-a	3' gauGGACGUGAUAUUCGUGAAAu 5' hsa-miR-20a 164:5' gacUCUGCGC-A-GAGCACUUUg 3' VEGFA	-0.139	0.7763
	HIF-1	3' gaUGGACGUGAUAUUCGUGAAAu 5' hsa-miR-20a 944:5' auGUUUG-AUUUUAUGCACUUUg 3' HIF1	-1.1634	0.7503
miR-20b	VEGF-a	3' gauGGACGUGAUACUCGUGAAAc 5' hsa-miR-20b 164:5' gacUCUGCGC-A-GAGCACUUUg 3' VEGFA	-0.139	0.7763
	HIF-1	3' gaUGGACGUGAUACUCGUGAAAc 5' hsa-miR-20b 944:5' auGUUUG-AUUUUAUGCACUUUg 3' HIF1	-1.1634	0.7503

Illustration: The predict-analysis was from the public data of net (www.microrna.org)

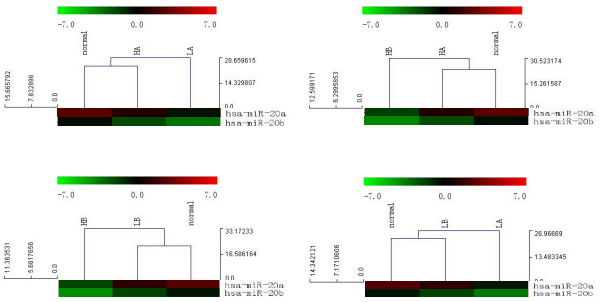

Figure 3. The Heat Map of the miR-20a and miR-20b

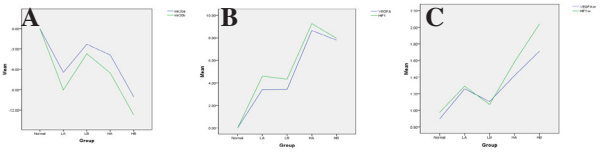

Figure 4. Verification Test by Realtime RT-qPCR & Western-Blot

in LB was -3.70±1.70, in HA was -6.55±2.21, and in HB was -12.72±2.18. The significant differences in the three groups are ranked as follows (P<0.05): HB<HA<Normal, LA<LB<Normal, HB<LB<Normal, and LA<HA<Normal (Figure 4).

Target Proteins as Predicted using Bioinformatic Tools

According to analysis using bioinformatics, vascular endothelial growth factor (VEGF)-A and hypoxia inducible factor-1 alpha (HIF-1alpha) are the target proteins regulated by miR-20a and miR-20b. HIF-1alpha can activate VEGF-A, thereby playing a role in the regulation of tumor angiogenesis.

Expression of VEGF-A and HIF-1alpha (mRNA and Protein) in Breast Tissue

The relative content of VEGF-A (mRNA) extracted from breast tissue in the normal group was 0.00±1.32, in LA was 3.40±1.56, in LB was 3.42±1.65, in HA was 8.65±1.27, and in HB was 7.79±1.18. The relative content of HIF-1 (mRNA) extracted from breast tissue in the normal group was 0.00±1.49, in LA was 4.59±1.87, in LB was 4.33±2.21, in HA was 9.25±0.88, and in HB was 8.00±2.35. The significant differences in the three groups are ranked as follows (P<0.05): Normal<LA<HA and Normal<LB<HB (Figure 4). The content of VEGF-A (protein) extracted from breast tissue in the normal group was 0.90±0.48, in LA was 1.26±0.75, in LB was 1.11±0.76, in HA was 1.42±0.47, and in HB was 1.71±1.21. The content of HIF-1alpha (protein) extracted from breast tissue in the normal group was 0.98±0.50,

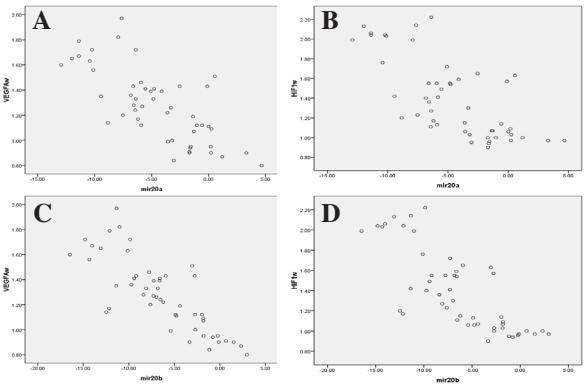

Figure 5. Scatter of Correlation Analysis

in LA was 1.29±0.10, in LB was 1.07±0.05, in HA was 1.58±0.07, and in HB was 2.04±0.12. The significant differences in the three groups are ranked as follows (P<0.05): Normal<LB<HB and Normal<LB<LA and Normal<HA<HB (Figure 4).

VEGF-A and HIF-1alpha Expression Inversely Correlates with miR-20a & miR-20b Expression in Breast Tissue

There was a significant negative correlation between miR-20a and VEGF-A protein extracted from breast tissue samples (Pearson correlation, r=-0.732, P<0.01), between miR-20a and HIF-1alpha protein (Pearson correlation, r=-0.722, P<0.01), between miR-20b and VEGF-A protein (Pearson correlation, r=-0.805, P<0.01), and between miR-20b and HIF-1alpha protein (Pearson correlation, r=-0.780, P<0.01) (Figure 5).

Discussion

In the past decade there have been remarkable advances in our understanding of microRNAs and their function in a large range of developmental and physiological processes (Hafez et al., 2012; Letonqueze et al., 2012). These small endogenous non-coding RNAs regulate hundreds of post-transcriptionally expressed genes, and recently, have been shown to play an important role in various human cancers (Nana-Sinkam and Croce, 2011; Piao and Ma, 2012). The center of solid tumors is hypoxic due to the uneven distribution of new blood vessels (Secomb et al., 2012). It is well-known VEGF plays a critical role in angiogenesis during both physiological and pathological processes (Kim et al., 2011). VEGF gradients in tissues are responsible for the initial triggering and guidance of the sprouting process (Gerhardt et al., 2003). In order to determine how key miRNAs are distributed and expressed in breast

cancer tumors, if there are differences in expression, and the relationship between these key miRNAs and VEGF regulation, we chose to analyze the center and edge of breast cancer specimens. Specifically, microRNA Array screening and verification tests were performed to see which patients had a higher metastatic rate clinically. MiR-20a and miR-20b were unevenly distributed in breast cancer, as confirmed by the microRNA-Array [Figure 3]. The relative expression of miR-20a and miR-20b was lower in the center of the tumor than at the edge in the LIMG, lower in the edge of the tumor than in the center in the HIMG, and lower in breast cancer tissues than in normal tissues (Figure 4). The differential expression of miR-20a between LIMG and HIMG (HB<LB<Normal) was opposite to the result in the research about ovarian and prostate (Fan et al., 2010; Pesta et al., 2010), and this related with differences in tumor-selective. According to our analysis using bioinformatic tools, we found that VEGF-A and HIF-1alpha were the target proteins regulated by both miR-20a and miR-20b (Table 5). VEGF-A and HIF-1alpha mRNA levels were higher in the HIMG than in the LIMG, and levels in both HIMG and LIMG were lower than in the Normal group; there were no differences in expression levels between the edge and center of the tumor (Figure 4). It is widely accepted that the higher the expression level of VEGF-A mRNA, the poorer the breast cancer prognosis (Linderholm et al., 2000). In addition, HIF-1alpha is an independent prognostic factor for an unfavorable prognosis in breast cancer patients with lymph node metastases (Schindl et al., 2002). HIF-1alpha is a master transcription factor that plays a key role in modulating the expression of various genes under hypoxia, and VEGF, a well-known target gene of HIF-1alpha, is induced by hypoxia through a HIF-1alpha independent pathway (Choi et al., 2011). VEGF-A and HIF-1alpha protein levels were higher in the HIMG than in LIMG, and were higher in the LIMG than in the Normal group; the significanct differences are ranked as LA<LB<Normal and HB<HA<Normal (Figure 4). These differences in protein expression in the breast cancer specimens were opposite to the expression of the miRNAs. Correlation analysis showed that expression of the key miRNAs (miR-20a and miR-20b) negatively correlated with the target proteins (VEGF-A and HIF-1alpha) (Figure 5). This negative correlation suggested that VEGF-A and HIF-1alpha may be regulated by miR-20a and miR-20b, which remains to be confirmed by future experiments in vitro. Undoubtedly, the higher the expression of VEGF-A and HIF-1alpha, the more invasion and metastasis there will be. However, the distribution of the target proteins in the tumor was completely opposite in HIMG and LIMG. As early as 1997, research by Guidi et al. confirmed that the expression of VEGF positively corelates with angiogenesis (Guidi et al., 1997). The author speculated that this was due to the following reasons: (1) when the expression of VEGF-A and HIF-1alpha in the center of tumor is higher than at the edges, this is conducive to local growth; (2) when the expression of VEGF-A and HIF-1alpha at the edge of tumor is higher than in the center, it is conducive to metastasis when; (3) the regulation of miR-20a and miR-20b at the level of translation is one of the most reliable reasons that causes the target proteins (VEGF-A and HIF-1alpha) expressing differentially in breast cancer. In summary, it appears that one of the most important facets underlying metastatic heterogeneity is the differential distribution of miR-20a and miR-20b and their regulation of expression of target proteins.

Acknowledgements

The author(s) declare that they have no competing interests.

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