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Screening miRNAs related to different subtypes of breast cancer with miRNAs microarray

E.-H. SUN, Q. ZHOU, K.-S. LIU, W. WEI, C.-M. WANG[1], X.-F. LIU, C. LU, D.-Y. MA

State key Laboratory of Reproductive Medicine, Department of Breast, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, China
[1]Department of Human Anatomy and Embryology, Nanjing University of Traditional Medicine, Xianlin Rd, Nanjing, China

Erhu Sun and *Qian Zhou* should be regarded as co-first authors

Abstract. – AIM: The aim of this study was to screen miRNAs related to different subtypes of breast cancer and their target genes to identify new markers of tumor subtype.

MATERIALS AND METHODS: The miRNA expression profiles of breast cancer GSE38867 including 7 ductal carcinoma in situ breast (DCIS) cancer samples, 7 invasive breast cancer samples, 7 metastatic breast cancer samples, and 7 normal breast samples) were downloaded from Gene Expression Omnibus (GEO) database. Limma package in R software was applied to identify specific differentially expressed miRNAs of different subtypes of breast cancer. MicroRNA.org database source was used to predict the target genes of the identified differentially expressed miRNAs. We integrated the target genes and their interacted genes (predicted by STRING) into DAVID to perform the GO function and KEGG pathway analyses.

RESULTS: Compared to the normal control, a total of 21, 47, and 107 differentially expressed miRNAs were screened in DCIS, invasive and metastatic breast cancer, respectively. Specific differentially expressed miRNAs of the three subtypes were identified, including hsa-miR-99a and hsa-miR-151-3p for DCIS breast cancer, hsa-miR-145 and hsa-miR-210 for invasive breast cancer, and has-miR-205 and has-miR-361-5p metastatic breast cancer. Furthermore, 220, 43, 446, 307, 587 and 328 interaction pairs of the specific miRNA targets were predicted. Multiple GO functions and KEGG pathways were enriched with the miRNA targets and their interacted genes.

CONCLUSIONS: We screened the most representative miRNAs of the three different subtypes of breast cancer, which may act as the putative markers in the diagnosis of different subtypes of breast cancer.

Key Words:
Breast cancer subtypes, miRNA, Target gene, Diagnosis.

Introduction

Breast cancer, like other cancers, occurs due to an interaction between the genetic and environmental factors[1]. The incidence of breast cancer is rapidly increasing in most Asian countries[2]. Prognosis and survival rates of breast cancer vary greatly and depend on the cancer types, stage, treatment, and geographical location of patient[3]. Histopathologic classification is based on the characteristics of biopsy specimens observed by light microscopy. The three most common histopathological subtypes which account for approximately three-quarters of breast cancers[2] are ductal carcinoma in situ breast cancer (DCIS), invasive breast cancer, and metastatic breast cancer[4].

Microarray technology has changed our understanding of prognosis and molecular classification of human cancers[5,6]. A DNA microarray analysis was performed to identify a gene expression signature which could predict the clinical outcomes of breast cancer[6]. The poor prognosis signature includes genes involved in cell cycle, invasion, metastasis, and angiogenesis. Besides, many researchers predicted that the phenotypic diversity of breast tumors might be accompanied by a corresponding diversity in gene expression patterns[7]. Systematic investigation of gene expression patterns in human breast tumors might help us to find the significant marker for different breast cancer subtypes[8]. miRNAs which have the potential to classify basal *vs.* luminal tumor subtypes have been reported[9]. However, none of specific miRNA signatures are identified to classify the DCIS, invasive, and metastatic breast cancer subtypes[9].

Hence, we analyzed the miRNA profiles of GSE38867, which include DCIS breast cancer, invasive breast cancer, and metastatic breast can-

Corresponding Author: Linmei Wang, MD; e-mail: wanglmeill@hotmail.com

cer specimens. As a result, six breast cancer-related miRNAs were screened out, including hsa-miR-145, hsa-miR-210, hsa-miR-151-3p, hsa-miR-99a, hsa-miR-205, and hsa-miR-361-5p. In addition, we selected the target genes of these miRNAs and performed the GO function and KEGG pathway analyses to investigate their mechanism in breast cancer.

Materials and Methods

Data source

The Affymetrix miRNA profiles of GSE38867 were download from National Center of Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), which is based on the GPL15019 (miRNA_V16.0_Microarray 030840). The dataset available in this study included 28 samples (7 DCIS breast cancer samples, 7 invasive breast cancer samples, 7 metastatic breast cancer samples, and 7 normal samples) (Table I).

Data preprocessing and differentially expression analysis

The original data were converted into expression measures. The deficient data were imputed[10], and the full data were normalized[11]. Then, we used the limma package of R language[12] to perform the differentially expression analyses (normal group *vs.* DCIS breast cancer group, normal group *vs.* invasive breast cancer group, and normal group *vs.* metastatic breast cancer group) to recognize the differentially expressed miRNAs between the different subtypes and normal tissue. The BH method was applied to perform a multiple test correction[13]. A p-value < 0.05, FDR < 0.05 and |logFC| > 1 were chosen as thresholds for screening the differentially expressed miRNAs.

Table I. Samples information.

	Sample Number	Age Range (years)
Normal breast tissue	7	28-62
DCIS breast cancer tissue	7	28-62
Invasive breast cancer tissue	7	28-62
Metastatic breast cancer tissue	7	28-62

DCIS, ductal carcinoma in situ.

Prediction of miRNA target genes

microRNA.org (http://www.microrna.org)[14], a comprehensive resource of microRNA target predictions, was used to predict the target genes of miRNAs using TargetScan[15], miRanda[16] and Pictar[17] algorithms. In order to reduce the false positive target prediction, we chose miRNA target genes at least in the two prediction algorithms as a high degree of confidence of miRNA target genes for subsequent analysis.

GO and KEGG pathway analysis

A single gene often does not play a role alone, but could interact with other molecules to play a regulatory role. The miRNA target genes were used to predict their interacted genes using STRING software[18]. The target genes and their interacted genes were used to perform interaction network map with cytoscape whose network function module be noted by GO annotation. We used the DAVID to identify significantly enriched GO functions and KEGG pathways using the genes in our result. A p-value < 0.05 and FDR < 0.05 were chosen as thresholds.

Results

Experimental procedure introduction

First of all, we used the limma package of R language to perform the differentially expression analysis between groups (normal group *vs.* DCIS breast cancer group, normal group *vs.* invasive breast cancer group, and normal group *vs.* metastatic breast cancer group). Combining with the three target genes of miRNA database, we selected differentially expressed miRNAs, and constructed the target genes network. Then in order to reveal the biological function of the target genes network, we predicted the function of miRNAs in the target genes network through enrichment of biological processes and biological pathways (Figure 1).

Selection of differentially expressed miRNAs

Using the limma package of R language, a total of 21, 47, and 107 differentially expressed miRNAs between normal group *vs.* DCIS breast cancer group, normal group *vs.* invasive breast cancer group, and normal group *vs.* metastatic breast cancer group were screened, respectively (Figure 2). Combined, nine common differentially expressed miRNAs in three subtypes of breast cancer compared to the normal group were identified and listed in Table II.

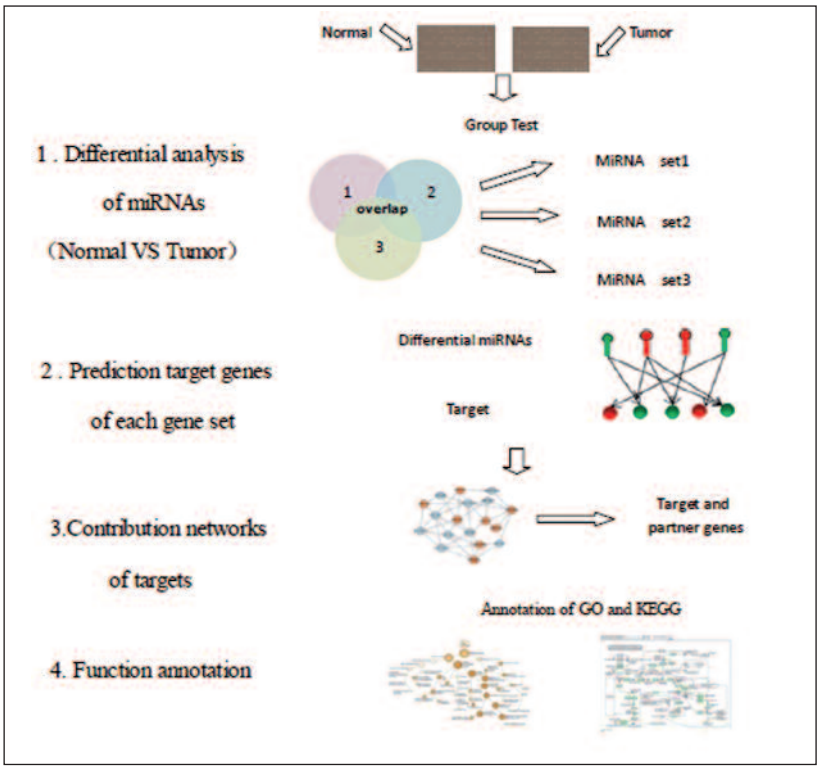

Figure 1. Diagram of prediction of putative breast cancer-related miRNAs based on the functional associations.

Analyze and identify the miRNAs

Nine common differentially expressed miRNAs were clustered according to their logFC values (Table II and Figure 3). Six specific differentially expressed miRNAs (down-regulated and up-regulated) in the three groups were shown in Table III. Among them, hsa-miR-145, hsa-miR-210, hsa-miR-99a and hsa-miR-151-3p were of the nine common differentially expressed miRNAs. Their logFC changes (Table II; represented by the changes of color in Figure 3) among the three subtypes were more than 1.

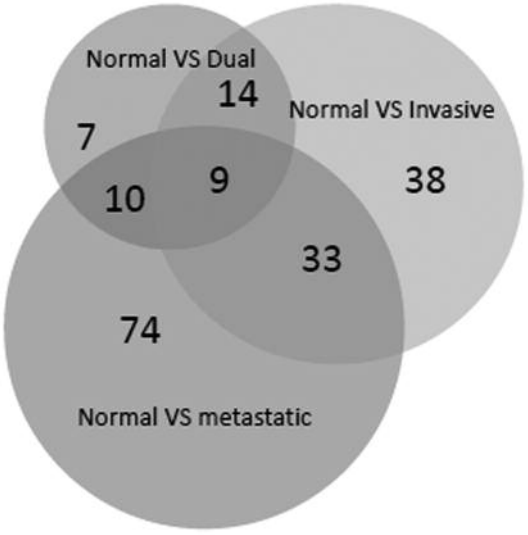

Figure 2. Intersection and union of three different genes groups.

Prediction of miRNAs target genes

In our study, target genes of the six specific differentially expressed miRNAs of the three subtypes were screened (Table IV). The breast cancer-related miRNAs of hsa-miR-145, hsa-miR-210, hsa-miR-151-3p, hsa-miR-99a, hsa-miR-205, and hsa-miR-361-5p had 12, 3, 10, 1, 3, and 11 target genes, respectively.

GO and KEGG pathway analysis

The miRNA target genes were used to predict their interacted genes using STRING software. The results illustrated that the breast cancer-related miRNAs of hsa-miR-145, hsa-miR-210, hsa-miR-151-3p, hsa-miR-99a, hsa-miR-205, and hsa-miR-361-5p showed 220, 43, 446, 307, 587, and 328 interactional pairs, respectively. GO function and KEGG pathway enrichment analyses of the target genes and their interacted genes were performed. The member of enriched GO terms and KEGG pathways were listed in Table V.

Table II. Nine common differetially expressed miRNAs in the three subtypes of breast cancer.

ID	dual_logFC	inva_logFC	meta_logFC
hsa-miR-125b	-3.8994	-4.87499	-4.96744
hsa-miR-145	-3.87523	-5.88435	-4.59057
hsa-miR-151-3p	5.36498	4.13822	3.58582
hsa-miR-196a	3.35724	3.73935	3.24739
hsa-miR-210	4.70087	2.56056	3.53268
hsa-miR-3675-3p	-3.51422	-3.08642	-3.43569
hsa-miR-4304	-2.49729	-2.8705	-2.5363
hsa-miR-492	3.96716	3.07072	3.09275
hsa-miR-99a	-4.32594	-4.57287	-3.90099

dual_logFC, logFC values of the differentially expressed miRNAs between the nomal group and DCIS breast cancer group; inva_logFC, logFC values of the differentially expressed miRNAs between the nomal group and invasive breast cancer group; meta_logFC, logFC values of the differentially expressed miRNAs between the nomal group and metastatic breast cancer group.

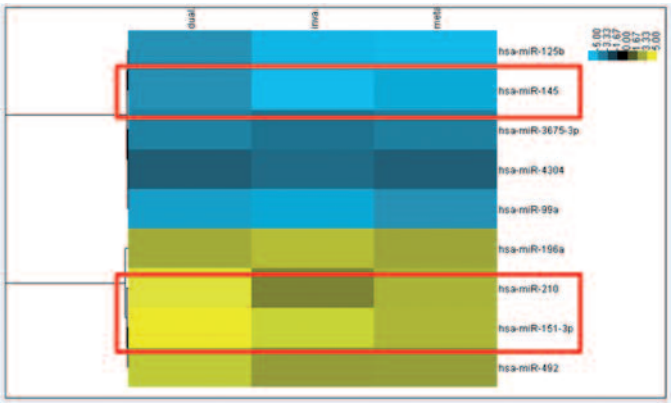

Figure 3. Cluster heat map of specific miRNAs of the three subtypes of breast cancer.

Table III. Specific differentially expressed miRNAs in the three subtypes of breast cancer.

Disease	Differentially expressed miRNA	
	Down-regulated	Up-regulated
DCIS breast cancer	hsa-miR-99a	has-miR-151-3p
Invasive breast cancer	has-miR-145	has-miR-210
Metastatic breast cancer	has-miR-205	has-miR-361-5p

DCIS, ductal carcinoma in situ.

Table IV. Target genes of the six specific miRNA.

miRNA	Target genes
hsa-miR-145	CTLA4, ITGB3, RAD23B, TGFBR2, GHRH, SRD5A2, TP53BP1, IGF1, IRS2, PTPRT, SRD5A2, ITGB3
hsa-miR-210	TFRC, TPMT, LTA
hsa-miR-151-3p	TIMP3, VDR, AHR, TGFBR2, CCND1, DMTF1, TP53, PTPRT, INSR, CALCR
hsa-miR-99a	RB1
hsa-miR-205	VEGEA, LRP1, PHB
hsa-miR-361-5p	VEGF1, MTUS1, RAD23B, MTR, GSR, TGFBR1, XRCC4, IL10, RB1, IGF1, MTRR

Table V. The enriched clusters and pathways.

miRNA	Num.of GO function clusters	Num. of KEGG pathway
hsa-miR-99a	104	11
has-miR-145	491	44
has-miR-205	222	23
has-miR-151-3p	535	42
has-miR-361-5p	223	19
has-miR-210	27	1

Discussion

Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk[19]. A number of screening test have been employed including: clinical and self breast exams, mammography, genetic screening, ultrasound, and magnetic resonance imaging[20]. In this study, we investigated the miRNA profiles of three different subtypes of breast cancer to find the significant miRNAs, hoping they can be used as the putative markers. Nine common miRNAs were clustered by logFC of differentially expression profiles. After a series analysis, we screened six sprcific miRNAs of different subtypes of breast cancer as our objects, that were hsa-miR-145, hsa-miR-210, hsa-miR-151-3p, hsa-miR-99a, hsa-miR-205, and hsa-miR-361-5p. In addition, all the genes related to the miRNA target genes were performed a function and pathway analysis, and the target genes of miRNAs and their interactional objects were converged into many functional clusters and pathways, which suggests the importance of these six miRNAs in breast cancer.

In our study, miR-145 was found to be down-regulated. Zhang et al[21] had reported that miRNA-145 is the substrate of Caspase-3 during apoptosis through experiments. Here, we verified that miRNA may be a tumor suppressor through Bioinformatics. The miR-210 has been strongly linked with the hypoxia pathway, and is up-regulated in response to Hypoxia-inducible factors[22]. Furthermore, it is also overexpressed in tumor cells[23]. So, we considered that the miR-210 is closely related to cancer. The miR-151, a frequently amplified miRNA, is correlated with intrahepatic metastasis of hepatocellular carcinoma[24]. It is also the significantly expressed genes in cancer. Recently, miR-99 has been found to suppress the expression of prostate-specific antigen and prostate cancer cell proliferation[25]. Our result suggested that the role of miRNA-99 in breast cancer was consistent to prostate cancer. Studies have demonstrated that miR-205 has a role in both normal development and cancer. In breast cancer, miR-205 was found down-modulated than that in normal breast tissue[26], consistent with our result. The miR-361 is a short RNA molecule, whose study was relatively less, our study confirmed that it was involved in up regulation of breast cancer.

Currently applied tumor markers in clinical breast cancer include CEA, CA15-3 and CA27.29, but their sensitivity and specificity are poor. Besides, it is difficult to effectively prompt the prognosis of patients at early diagnosis. Tumor metastasis is the main reason for the death of breast cancer patients[27]. New research shows that some miRNAs regulate post-transcriptional level of breast cancer metastasis-related gene expression, and are closely related to breast cancer invasion and metastasis[28]. At present, breast cancer and breast cancer metastasis-related miRNA research has been going on. The researchers adopted a variety of research tools, including microRNA microarray technology (LC Sciences) to conduct in-depth study of human cancer metastasis regulator, and found a series of abnormal expression of microRNAs closely related to breast cancer metastasis.

Conclusions

This analysis reported the miRNAs which could be used as the markers of different subtypes of breast cancer. The miRNAs and their target genes have very important functions, and play an important role in the classification and diagnosis of breast cancer subtypes. However, we predicted the target genes of miRNAs, through the integration of the database algorithm, further validation is necessary.

Conflict of interest

The Authors declare that they have no conflict of interests.

References

1) Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad USA 2003; 100: 3983-3988.
2) Tavassoli FA, Devilee P. Pathology and genetics of tumours of the breast and female genital organs. World Health Organization, 2003.
3) Boyle P, Levin B. World cancer report 2008. IARC Press, Int Agency Res Cancer, 2008.
4) Sant M, Allemani C, Capocaccia R, Hakulinen T, Aareleid T, Coebergh JW, Coleman MP, Grosclaude P, Martinez C, Bell J. Stage at diagnosis is a key explanation of differences in breast cancer survival across Europe. Int J Cancer 2003; 106: 416-422.
5) Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci USA 2003; 100: 10393-10398.

6) van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002; 415: 530-536.

7) Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA. Molecular portraits of human breast tumours. Nature 2000; 406: 747-752.

8) Hu Z, Fan C, Oh D, Marron J, He X, Qaqish B, Livasy C, Carey L, Reynolds E, Dressler L. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 2006; 7: 96.

9) Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavare S, Caldas C, Miska EA. Microrna expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 2007; 8: R214.

10) Troyanskaya O, Cantor M, Sherlock G, Brown P, Hastie T, Tibshirani R, Botstein D, Altman RB. Missing value estimation methods for DNA microarrays. Bioinformatics. 2001; 17: 520-525.

11) Fujita A, Sato JR, Rodrigues LO, Ferreira CE, Sogayar MC. Evaluating different methods of microarray data normalization. BMC Bioinformatics 2006; 7: 469.

12) Gentleman R. Bioinformatics and computational biology solutions using r and bioconductor. Springer Verlag, 2005.

13) Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Roy Stat Soc B Met 1995: 289-300.

14) Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microrna. Org resource: Targets and expression. Nucleic Acids Res 2008; 36: D149-D153.

15) Lewis BP, Shih I, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microrna targets. Cell 2003; 115: 787-798.

16) John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human microrna targets. PLoS Biology 2004; 2: e363.

17) Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, Da Piedade I, Gunsalus KC, Stoffel M. Combinatorial microrna target predictions. Nat Genet 2005; 37: 495-500.

18) Da Wei Huang BTS, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. Nat Protoc 2008; 4: 44-57.

19) Sariego J. Breast cancer in the young patient. Am Surg 2010; 76: 1397-1400.

20) Toi M, Ohashi Y, Seow A, Moriya T, Tse G, Sasano H, Park BW, Chow LWC, Laudico AV, Yip CH. The breast cancer working group presentation was divided into three sections: The epidemiology, pathology and treatment of breast cancer. Jpn J Clin Oncol 2010; 40: i13-i18.

21) Zhang J, Guo H, Qian G, Ge S, Ji H, Hu X, Chen W. Mir-145, a new regulator of the DNA fragmentation factor-45 (dff45)-mediated apoptotic network. Mol Cancer 2010; 9: 211.

22) Tsuchiya S, Fujiwara T, Sato F, Shimada Y, Tanaka E, Sakai Y, Shimizu K, Tsujimoto G. Microrna-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (fgfrl1). J Biol Chem 2011; 286: 420-428.

23) Li T, Cao H, Zhuang J, Wan J, Guan M, Yu B, Li X, Zhang W. Identification of mir-130a, mir-27b and mir-210 as serum biomarkers for atherosclerosis obliterans. Clin Chim Acta 2011; 412: 66-70.

24) Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, Ge C, Yao J, Chen T, Wan D. Gain of mir-151 on chromosome 8q24. 3 facilitates tumour cell migration and spreading through downregulating rhogdia. Nat Cell Biol 2010; 12: 390-399.

25) Sun D, Lee YS, Malhotra A, Kim HK, Matecic M, Evans C, Jensen RV, Moskaluk CA, Dutta A. Mir-99 family of microrna suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res 2011; 71: 1313-1324.

26) Greene SB, Herschkowitz JI, Rosen JM. The ups and downs of mir-205: Identifying the roles of mir-205 in mammary gland development and breast cancer. RNA Biology 2010; 7: 300-304.

27) Negrini M, Calin GA. Breast cancer metastasis: A microrna story. Breast Cancer Res. 2008; 10: 203.

28) Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microrna-10b in breast cancer. Nature 2007; 449: 682-688.