RESEARCH ARTICLE

Characterization of the MicroRNA Expression Profile of Cervical Squamous Cell Carcinoma Metastases

Hui Ding[1], Yi-Lin Wu[1]*, Ying-Xia Wang[2], Fu-Fan Zhu[1]

Abstract

Objectives: MicroRNAs (miRNAs) are important regulators of many physiological and pathological processes, including tumorigenesis and metastasis. In this study, we sought to determine the underlying molecular mechanisms of metastatic cervical carcinoma by performing miRNA profiling. Methods: Tissue samples were collected from ten cervical squamous cancer patients who underwent hysterectomy and pelvic lymph node (PLN) dissection in our hospital, including four PLN-positive (metastatic) cases and six PLN-negative (non-metastatic) cases. A miRNA microarray platform with 1223 probes was used to determine the miRNA expression profiles of these two tissue types and case groups. MiRNAs having at least 4-fold differential expression between PLN-positive and PLN-negative cervical cancer tissues were bioinformatically analyzed for target gene prediction. MiRNAs with tumor-associated target genes were validated by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Results: Thirty-nine miRNAs were differentially expressed (>4-fold) between the PLN-positive and PLN-negative groups, of which, 22 were up-regulated and 17 were down-regulated. Sixty-nine percent of the miRNAs (27/39) had tumor-associated target genes, and the expression levels of six of those (miR-126, miR-96, miR-144, miR-657, miR-490-5p, and miR-323-3p) were confirmed by quantitative (q)RT-PCR. Conclusions: Six MiRNAs with predicted tumor-associated target genes encoding proteins that are known to be involved in cell adhesion, cytoskeletal remodeling, cell proliferation, cell migration, and apoptosis were identified. These findings suggest that a panel of miRNAs may regulate multiple and various steps of the metastasis cascade by targeting metastasis-associated genes. Since these six miRNAs are predicted to target tumor-associated genes, it is likely that they contribute to the metastatic potential of cervical cancer and may aid in prognosis or molecular therapy.

Keywords: Cervical squamous cell carcinoma - miRNA - miRNA microarray - quantitative RT-PCR - target gene

Asian Pac J Cancer Prev, **15 (4)**, 1675-1679

Introduction

Cervical cancer is one of the most commonly diagnosed gynecological cancers. According to the IARC GLOBOCAN worldwide cancer incidence and mortality database (http://globocan.iarc.fr/fact sheets/cancers/cervix.asp), 530 000 new cases were reported in 2008. Developing countries accounted for more than 85% of the global burden, with China contributing around 75 000 of those cases. Detection of cancer cells in the lymph node (LN) is widely-recognized as one of the most important indicators of cervical cancer prognosis and guides the design of appropriate treatment strategies. Yet, the underlying molecular mechanisms that mediate the metastatic process leading to positive LN status in cervical cancer patients have yet to be fully elucidated.

The recently identified regulatory mechanism of microRNA (miRNA)-mediated post-transcriptional gene expression has emerged as an intriguing possible regulator of cervical cancer metastasis. MiRNAs are small noncoding RNAs of 18-25 nucleotides in length, which bind to the 3'-untranslated region (UTR) of target mRNAs. This sequence-specific binding leads to target mRNA degradation or repression of its translation, consequently decreasing encoded protein levels (Lee et al., 1993; Bagga et al., 2005). In fact, miRNAs have already been implicated as regulators of many physiological and pathological processes, including development, proliferation, differentiation, apoptosis, and tumor progression and metastasis (Bartel et al., 2004; He et al., 2004; Esquela-Kerscher et al., 2006; Negrini et al., 2008; Tavazoie et al., 2008; Bartels et al., 2009; Garzon et al., 2009). However, the role of miRNAs in cervical cancer metastasis and the precise mechanism of its action in these tumor types remain largely unexplored.

Therefore, this study was designed to identify the specific miRNAs that are associated with cervical squamous cancer metastasis to the lymph node. A panel of miRNAs discovered by initial microarray screening of tumors from LN-positive and LN-negative cervical

*[1]Department of Gynecology, Second Xiangya Hospital, Central-South University, [2]Department of Woman Health Care, Maternal and Child Health Hospital of Hunan Province, Changsha, China *For correspondence: chshwuyilin@sina.cn*

cancer patients was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The predicted target genes of these significantly differentially expressed miRNAs were then investigated to determine the potential carcinogenic mechanisms.

Materials and Methods

Specimens

We collected tumor samples from 10 cervical squamous carcinoma patients who underwent hysterectomy with LN dissection at the Department of Gynecology, Second Xiangya Hospital of Central-South University between July and October of 2010. All patients provided written informed consent, and the study was approved by the Institutional Ethics Committee. None of the patients had received preoperative chemotherapy or radiotherapy. Four of the patients were confirmed as pelvic lymph node (PLN)-positive, indicating metastasis. Of the remaining 6 patients that were confirmed as being PLN-negative (without metastasis), and were selected for use in the comparative analysis.

The total 10 patients used in the study ranged in age from 29 to 65 years old (median: 41.5 years). The patients were staged according to the Federation of Gynecology and Obstetrics (FIGO) 2000 staging system, by which five were classified as Ib1, one as Ib2, and four as IIa. The patients' histological findings (hematoxylin and eosin (H&E) stain) indicated that nine were of middle-grade and one was of low-grade. The PLN-negative group and PLN-positive group had no significant difference in stage, degree of differentiation, age, tumor size, or HPV infected status.

One-half of each tissue sample collected was divided into two tubes, frozen in liquid nitrogen immediately after surgical removal, and stored at -80°C.

MiRNA microarray analysis

One of the two frozen tissue samples from each patient was submitted to KangChen-Biotech (Shanghai, China) for miRNA microarray analysis. At KangChen-Biotech, the total RNA (including miRNA) was isolated from individual samples by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The purity and quantity of the isolated RNAs were assessed by 1% formaldehyde-agarose gel electrophoresis and spectrophotometry (Bio-Rad, Hercules, CA, USA). The RNAs from PLN-positive group and PLN-negative group have been respectively mixed and put into microarray analysis. After further processing, the isolated miRNAs were labeled with Hy3 by using the miRCURY Hy3/Hy5 Power labeling kit (Exiqon, Vedbaek, Denmark). The labeled miRNAs were then hybridized to individual miRCURYTM LNA microRNA Arrays (v 14.0; Exiqon), as described previously (Castoldi et al., 2006). Microarray images were acquired by an Axon Genepix 4000B microarray scanner (Axon Instruments, Union City, CA, USA), and processed and analyzed by the accompanying Axon Genepix Pro 6.0 software.

MiRNAs were considered as differentially expressed between cervical cancer tissues with lymph node metastasis and without lymph node metastasis if the difference reached at least 4-fold.

Table 1. MiRNA-specific Primers Used in the qRT-PCR

miRNA	Forward primer (5'-3')
U6	UUCACGAAUUUGCGUGUCAU
hsa-miR-490-5p	CATGGATCTCCAGGTGGGT
hsa-miR-657	GCAGGTTCTCACCCTCTCTAGG
hsa-miR-323-3p	CACATTACACGGTCGACCTCT
hsa-miR-144	GCCTACAGTATAGATGATGTACT
hsa-miR-96	CGTTTGGCACTAGCACATTTTTG
hsa-miR-126	TCGTACCGTGAGTAATAATGCG

hsa, homo sapien

Bioinformatic prediction of miRNAs target genes

The differentially expressed miRNAs were investigated to predict their potential target genes by using the iterative algorithms of TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de), and MiRanda (http://mirdb.org/miRDB/). Target genes were selected based on prediction by at least two of the programs. The identified genes were further selected for those with functions previously associated to tumor processes.

QRT-PCR validation

Six of the miRNAs that had significantly different expression levels according to the microarray analysis and had tumor-associated target genes were selected for verification by qRT-PCR. The qRT-PCR assays were performed on a QPCR System (Applied Biosystems, Inc., Foster City, CA, USA) using RNA that had been Trizol-extracted in our lab from the second frozen tissue sample. The isolated RNA was quantified by UV-spectroscopy and used as template to synthesize cDNA with the One Step PrimeScript miRNA cDNA Synthesis kit (TaKaRa, Dalian, China).

The qPCR assay was carried out with reagents from the SYBR Green Real-Time PCR kit (TaKaRa), and the reaction mix was composed of 10 μL SYBR Premix Ex Taq II, 0.8 μL PCR forward primer (Table 1), 0.8 μL Uni-miR qPCR primer , 2 μL cDNA, 0.4 μL ROX reference dye, and 6 μL deionized H2O. Reactions were incubated in a 48-well optical plate at 95˚C for 30 s, followed by 40 cycles of 95˚C for 5 s, and 60˚C for 34 s.

The threshold cycle (Ct) was determined by using the default threshold settings. All experiments were carried out in triplicate and repeated three times. U6 was used as an endogenous control, to which miRNA expression was normalized. Normalized data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The results were then calculated as fold-change of each miRNA detected in the cervical cancer tissues with lymph node metastasis relative to that in the tissues without lymph node metastasis.

Statistical analysis

Statistical data analyses were performed with the rank sum test, using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA), and p-values <0.05 were considered significant.

Table 2. MiRNAs Differentially Expressed in Cervical Cancer Tissues with Lymph Node Metastasis as Compared to Cervical Cancer Tissues Without Lymph Node Metastasis

miRNA increased	fold-change	miRNA decreased	Fold-change
hsa-miR-490-5p	780.34	hsa-miR-652	-22.67
hsa-miR-323-3p	220.96	hsa-miR-144	-13.64
hsa-miR-675*	78.63	hsa-miR-96	-10.85
hsa-miR-657	36.58	hsa-miR-135b	-10.70
hsa-miR-551a	9.20	hsa-miR-181d	-10.66
hsa-miR-550a*	8.99	hsa-miR-377	-8.91
hsa-miR-1291	8.96	hsa-miR-30a	-8.19
hsa-miR-328	8.88	hsa-miR-93	-7.38
hsa-miR-326	8.39	hsa-miR-30c	-6.32
hsa-miR-489	8.38	hsa-miR-126	-6.25
hsa-miR-585	7.62	hsa-miR-455-5p	-5.82
hsa-miR-1184	7.53	hsa-miR-1285	-5.33
hsa-miR-639	7.49	hsa-miR-210	-5.18
hsa-miR-488	6.09	hsa-miR-191	-5.15
hsa-miR-612	5.90	hsa-miR-27a	-4.77
hsa-miR-1204	5.51	hsa-miR-23a*	-4.57
hsa-miR-941	5.34	hsa-miR-31	-4.38
hsa-miR-208b	5.30		
hsa-miR-206	5.23		
hsa-miR-219-1-3p	4.72		
hsa-miR-576-5p	4.34		
hsa-miR-374b*	4.05		

Results

Significant differentially expressed miRNAs

Of the 1223 miRNA genes analyzed by miRNA microarray, 39 were identified as being differentially expressed by more than 4-fold. Of those, 22 were up-regulated and 17 were down-regulated in cervical carcinomas with LN metastasis (Table 2).

Predicted target genes of the differentially expressed miRNAs in metastatic cervical cancer

By using a combined approach of analyzing the miRNA sequence by bioinformatic neural nets and searching previously published studies regarding the particular genes' functions, we were able to predict tumor-associated gene targets of the significantly differentially expressed 39 miRNAs. Twenty-seven of those miRNAs (69%) were found to have tumor-associated putative target genes, including 17 of the up-regulated miRNAs and 10 of the down-regulated miRNAs (data not shown). Results from subsequent verification analysis, narrowed the list to six of those 27 miRNAs, which are summarized in Table 3.

Validation of microarray data by qRT-PCR analysis

The differentially expressed miRNAs detected on the

Table 3. Putative Target Genes of the Six Differentially Expressed miRNAs

miRNA	Target gene	Molecular function
miR-490-5p	MMP16	Metastasis
	RPS6KA3	Cell growth and differentiation
	FOS	Cell proliferation, differentiation, transformation, and apoptotic cell death
miR-323-3p	APPL1	Cell proliferation
	KRAS	Implicated in pathogenesis of various malignancies, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas and colorectal carcinoma
	LPHN3	Cell adhesion and signal transduction
miR-657	CDC14B	Cell cycle control Regulate the function of p53
	NAP1L	Cell proliferation
	TNFSF11	Cell apoptosis
	PTPN2	Cell growth, differentiation, mitotic cycle, and oncogenic transformation
miR-126	PTPN9	Cell growth, differentiation, mitotic cycle, and oncogenic transformation
	CRK	Tumor migration, metastasis, and proliferation
	PLXNB2	Cell migration
miR-96	FOXO1	Cell proliferation and apoptosis
	FN1	Cell adhesion and migration
	EZRin	Cell surface structure adhesion, migration, and organization
	KRAS	Implicated in pathogenesis of various malignancies, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas and colorectal carcinoma
	E2F5	Control of cell cycle Regulate action of tumor suppressor proteins
	CD164	Adhesion receptors
	SDC2	Cell proliferation, cell migration, and cell-matrix interactions via its receptor for extracellular matrix proteins
	SH3KBP1	Apoptosis, cytoskeletal rearrangement, and cell adhesion
	PIK3C2A	Cell proliferation, oncogenic transformation, cell survival, and cell migration
miR-144	FN1	Cell adhesion and migration
	CXCL12	Implicated in the metastasis of some cancers, such as breast cancer
	PTPN9	Cell growth, differentiation, mitotic cycle, and oncogenic transformation
	MMP16	Metastasis
	ETS1	Cell development, cell senescence, cell death, and tumorigenesis
	CRK	Tumor migration, metastasis, and proliferation

Table 4. Comparison of the Expression Levels of miRNAs Detected on Microarray and by Quantitative RT-PCR

miRNA	Microarray	qRT-PCR
miR-490-5p	780.34	56.90
miR-323-3p	220.96	8.18
miR-657	36.58	9.86
miR-144	-13.64	-32.47
miR-96	-10.85	-16.58
miR-126	-6.25	-9.72

miRNA microarray were found to have tumor-associated putative target genes, and six of these were verified by quantitative real-time-PCR (Table 4). We select these six miRNAs to testify whether they do express differently is that, of the research on target proteins of these six miRNAs, many have proved that these proteins impact on the tumor invasion and metastasis. MiR-126, miR-96, and miR-144, which were detected on microarray as being down-regulated in metastatic cervical cancer, also showed significantly lower expression than in non-metastatic cervical cancer by qRT-PCR. Likewise, the miR-657, miR-490-5p, and miR-323-3p, which were detected as up-regulated by the microarray experiments, were found by qRT-PCR to be significantly up-regulated.

Discussion

Although several miRNAs have been found to function as either tumor metastasis promoters or suppressors (Asangani et al., 2008; Korpal et al., 2008; Li et al., 2009; Tsai et al., 2009), the exact role that miRNAs play in cervical cancer metastasis is only beginning to be uncovered. Here, we describe our microarray-based investigation into the metastatic-related miRNA expression profile of cervical squamous cancer. Among these differently expressed 39 miRNAs, 27 had predicted target genes whose functions are known to be closely associated with tumor pathogenesis.

Quantitative RT-PCR experiments verified the differential expression microarray findings for the down-regulated miR-126, miR-96, and, miR-144, and the up-regulated miR-657, miR-490-5p, and miR-323-3p. Thus, our findings indicate that miRNA might play an important role in the metastasis of cervical squamous cancers.

In our study, the verified pattern of miR-126 down-regulation in metastatic cervical cancer specimens suggested a possible tumor metastatic suppressor function for this particular miR-126 in cervical cancer. Previous research of colon cancer (Li et al., 2013), cervical cancer (Yu et al., 2013) and gastric cancers (Feng et al., 2010) also observed down-regulated expression of miR-126. Moreover, it has been shown that retrovirus-mediated restoration of miR-126 expression in murine primary breast cancer cells caused the suppression of overall tumor growth and metastasis to the bone and lung (Sohail et al., 2008). Our data from cervical cancer specimens are consistent with these previous studies, and support the theory that miRNAs, particularly miR-126, contribute to the underlying molecular mechanisms of tumor metastasis.

Since we were interested in gaining a more detailed understanding of the underlying molecular mechanisms of metastasis, we investigated the predicted target genes of the significantly deregulated miRNAs: miR-126, miR-96, miR-144, miR-657, miR-490-5p, and miR-323-3p. Not surprisingly, our initial approach using bioinformatic neural nets to predict target genes based on corresponding sequence pairing yielded hundreds of genes for each miRNA (Li et al., 2007). It is likely that miRNAs may specifically target a variety of genes according to the precise physiological context or tissue-specific intracellular milieu. In fact, it has been reported that almost one-third of the protein-coding genes in humans are susceptible to miRNA regulation (Lewis et al., 2005) and, as a consequence, identification of one or several important targets may provide useful insights into the context- and tissue-specific functions of certain miRNAs.

We, therefore, focused our research efforts on the miRNAs with target genes that had been previously related to tumor pathogenesis in the peer-reviewed literature. Thus, six MiRNAs with predicted tumor-associated target genes were identified. The target genes of these miRNAs encoded proteins that are known to be involved in cell adhesion, cytoskeletal remodeling, cell proliferation, cell migration, and apoptosis. These findings suggest that a panel of miRNAs may regulate multiple and various steps of the metastasis cascade by targeting metastasis-associated genes. In support of this theory, a previous study by Valastyan et al. had shown that alteration of miR-31 modulated multiple target genes and affected multiple phenotypes, such as motility, invasion, and resistance to anoikis (Valastyan et al., 2009). Pacurari M et al. reported the miR-200 family and these potential targets were functionally involved in canonical pathways of immune response, molecular mechanisms of cancer, metastasis signaling, cell-cell communication, proliferation and DNA repair in Ingenuity pathway analysis (Pacurari et al., 2013). Multiple-to-multiple relationships between miRNAs and targets have been reported by transfection experiments involving human gastric cancer cells (Hashimoto et al., 2013). Therefore, we speculate that miRNAs regulation of multiple and various target genes contributes to cervical cancer metastasis.

In conclusion, we used miRNA microarray technology to analyze the different miRNA expression profiles between cervical cancer specimens with lymph node metastasis and without lymph node metastasis, and 39 significantly disregulated miRNAs were identified. The expression levels of miR-126, miR-96, miR-144, miR-657, miR-490-5p, and miR-323-3p were validated by qRT-PCR, and the results agreed with the patterns detected by microarray analysis. Target gene predictions indicated that differentially expressed miRNAs may play an important role in the metastasis of cervical cancer, and that identified specific tumor-associated genes may be involved in the pathogenic process. However, this set of experiments remain some insufficiencies, such as tumor specimens from patients were fewer; 39 miRNAs were identified by miRNA microarray as being differentially expressed by more than 4-fold, we only select these six miRNAs to testify. So we need to expand the sample to estimate the value of miRNAs for predicting lymph node

metastasis of cervical squamous cell carcinoma. Further investigative focus on these miRNAs and their functions in the pathogenesis of cervical cancer will be necessary.

References

Asangani IA, Rasheed SA, Nikolova DA, et al (2008). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene*, **27**, 2128-36.

Bagga S, Bracht J, Hunter S, et al (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, **122**, 553-63.

Bartels CL, Tsongalis GJ (2009). MicroRNAs: novel biomarkers for human cancer. *Clin Chem*, **55**, 623-31.

Bartel DP (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-97.

Castoldi M, Schmidt S, Benes V, et al (2006). A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA*, **12**, 913-20.

Esquela-Kerscher A, Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, **6**, 259-69.

Feng R, Chen X, Yu Y , et al (2010). MiR-126 functions as a tumour suppressor in human gastric cancer. *Cancer Lett*, **298**, 50-63.

Garzon R, Calin GA, Croce CM (2009). MicroRNAs in Cancer. *Annu Rev Med*, **60**, 167-79.

Hashimoto Y, Akiyama Y, Yuasa Y (2013). Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer. *PLoS One*, **8**, e62589.

He L, Hannon GJ (2004). MicroRNAs: Small RNAs with a big role in gene regulation. *Nat Rev Genet*, **5**, 522-31.

Korpal M, Lee ES, Hu G, et al (2008). The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem*, **283**, 14910-4.

Lee RC, Feinbaum RL, Ambros V (1993). The C. elegans heterochomic gene lin-4 encodes small RNAs with antisense complematarily to lin-4. *Cell*, **75**, 843-54.

Lewis BP, Burge CB, Bartel DP (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15-20.

Li N, Tang A, Huang S, et al (2013). MiR-126 suppresses colon cancer cell proliferation and invasion via inhibiting RhoA/ROCK signaling pathway. *Mol Cell Biochem*, **380**, 107-19.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-Delta Delta Ct method. *Methods*, **25**, 402-8.

Li SC, Tang P, Lin WC (2007). Intronic microRNA: discovery and biological implications. *DNA Cell Biol*, **26**, 195-207.

Li T, Li D, Sha J, et al (2009). MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. *Biochem Biophys Res Commun*, **383**, 280-5.

Negrini M, Calin G A (2008). Breast cancer metastasis: a microRNA story. *Breast Cancer Res*, **10**, 203.

Pacurari M, Addison JB, Bondalapati N, et al (2013). The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells. *Int J Oncol*, **43**, 548-60.

Sohail F, Tavazoie, Alarcón C, Oskarsson T (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*, **451**, 147-52.

Tavazoie SF, Alarco´n C, Oskarsson T, et al (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*, **451**, 147-52.

Tsai WC, Hsu PW, Lai TC et al (2009). MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. *Hepatology*, **49**, 1571-82.

Valastyan S, Reinhardt F, Benaich N, et al (2009). A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell*, **137**, 1032-46.

Yu Q, Liu SL, Wang H, et al (2013). MiR-126 suppresses the proliferation of cervical cancer cells and alters cell sensitivity to the chemotherapeutic drug bleomycin. *Asian Pac J Cancer Prev*, **14**, 6569-72