microRNA expression profiles in human colorectal cancers with brain metastases

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Abstract. The present study aimed to identify microRNA (miRNA) expression profiles associated with brain metastases of colorectal cancers. We conducted miRNA expression profiling of 3 primary colorectal cancers and 3 brain-metastatic carcinomas using Agilent Human miRNA Microarrays. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was employed to validate the results obtained from the miRNA microarray analysis. Overexpression of miR-145, miR-1, miR-146a, miR-576-5p, miR-126*, HS287, miR-28-5p, miR-143, miR-199b-5p, miR-199a-5p, miR-10b, miR-22, miR-133b, miR-145*, miR-199a, miR-133a, miR-125b and downregulation of miR-31 and HS170 were observed in brain-metastatic carcinomas. Quantitative RT-PCR experiments with miR-125b confirmed the expression patterns we found in our microarray experiments. miRNAs are differentially expressed between colorectal cancers and matched brain-metastatic carcinomas. The miRNA variation trend is quite different in the process of metastasis compared to that of carcinogenesis. These miRNAs may therefore serve as potential diagnostic markers and therapeutic targets for colorectal cancers with brain metastases.

Introduction

Colorectal cancer (CRC) is one of the most common digestive tract malignancies worldwide. The incidence of CRC is lower in China than in Western countries, but has increased in recent years due to changes in lifestyle and nutritional habits. CRC is currently one of the most serious threats to human health in China (1). Over the past decade, newer and more effective systemic therapies have been applied in advanced CRC patients. Active chemotherapeutic agents (specifically capecitabine, oxaliplatin and irinotecan) and biological drugs (such as cetuximab and bevacizumab) have provided further significant benefits (2). Due to marked advances in life prolongation, the liver and lungs are no longer exclusive targets among potential metastatic sites, and the incidence of metastases at previously uncommon sites, such as the brain, is on the increase (3). Brain metastases are relatively rare in CRC, with a reported incidence ranging from <1 to 4%. Furthermore, newer neuroimaging modalities have superior accuracy in identifying small (<5 mm) asymptomatic brain lesions (4). Therefore, CRC with brain metastasis requires careful consideration.

Gene expression profiling in CRC has been extensively studied in recent years. In the majority of studies, differences in gene expression were observed between normal mucosa and CRC tissues. Certain investigators studied metastasis-related genes by comparing CRC and corresponding metastases from the same patients, and found that expression levels of the majority of differentially expressed genes varied moderately (5,6). These findings were in accordance with the theory, proposed by Bernards and Weinberg (7), that there are no genes or genetic changes that are specifically and exclusively involved in the process of metastasis. Instead, the combination of oncogenic alterations, rather than particular events, determines the metastatic genotype of tumor cells early in tumorigenesis.

Mounting evidence indicates that microRNAs (miRNAs) may be key players in the regulation of tumor cell invasion and metastasis (8). miRNAs are a large family of small non-coding RNAs that negatively control gene expression at the mRNA and protein levels. Mature miRNAs comprise 19-25 nucleotides that silence genes through consummate or defective binding to the 3' untranslated region of the transcript. Microarray studies have revealed that individual miRNAs are capable of affecting the expression of multiple genes, indicating that miRNAs may have pleiotropic effects on cellular processes including cell differentiation, proliferation and apoptosis (9,10). An increasing number of studies have indicated that there are differential miRNA expression profiles between those in cancer tissues and adjacent non-tumorous tissue (11-14). However, few studies of metastasis-related miRNAs have compared CRC and matched metastatic carcinomas.

As has been established, metastasis, the colonization of tumor cells in another site discontinuous from the primary CRC, is the major cause of death for CRC patients (15). To understand the molecular mechanism of CRC with brain

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Table I. Clinicopathological features of 4 paired primary and metastatic CRCs.

Case No.	Gender	Age	Tumor size, cm		Histological differentiation		Primary site	Metastatic site
			P	M	P	M		
1	M	46	4.5	2.0	Poor	Poor	Sigmoid	Right parietal lobe
2	M	59	6.0	3.0	Moderate	Moderate	Descending	Cerebella
3	F	56	4.0	2.5	Moderate	Poor	Sigmoid	Left frontal lobe
4	F	54	6.0	4.0	Poor	Poor	Ascending	Left frontal lobe

P, primary tumor; M, metastatic tumor.

metastases, we used microarrays to directly characterize the molecular basis of metastases. We then selected 3 primary CRC tissues and matched metastatic carcinomas from the brain, and compared the miRNA expression profiles.

Materials and methods

Patients and tissue specimens. Primary CRC samples were collected from 4 patients who underwent radical resection of CRC in Huashan Hospital of Fudan University from May 2005 to November 2008. Their matched metastatic carcinomas from the brain were resected within 3 months prior or subsequent to the digestive tract surgery. Craniotomy and radical surgery for CRC were performed in our hospital to allow collection of the samples of CRC and brain-metastatic carcinomas. Tissues were snap-frozen in liquid nitrogen following surgical resection and stored at -80˚C until RNA extraction. Clinical information was collected from patient records. None of the CRC patients had received chemotherapy prior to surgery. Written informed consent was obtained from all CRC patients or their guardians. This study was performed according to the Committee for the Conduct of Human Research of Huashan Hospital of Fudan University.

RNA isolation. Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The RNA quantity was then determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The samples were assayed and qualified in triplicate using the miRNA microarray.

miRNA microarray profiling. The input for the Agilent miRNA labeling system was 100 ng total RNA. After dephosphorylation and denaturation, the total RNA was labeled with cyanine 3-pCp and then hybridized to Agilent Human miRNA Microarray V2.0 using the miRNA Complete Labeling and Hyb Kit (Agilent). Following hybridization for 20 h, the slides were washed using the Gene Expression Wash Buffer Kit (Agilent) and scanned using an Agilent Scanner. The images were processed and analyzed with Agilent Feature Extraction Software. The raw data were normalized using quantile normalization and then analyzed in GeneSpring GX software (zcomSilicon Genetics, Redwood City, CA, USA). Statistical analysis using ANOVA was conducted to compare the differentially expressed miRNAs.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). A cDNA synthesis was carried out using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A quantitative PCR was performed using a miScript SYBR-Green PCR Kit (Qiagen). Expression analysis was performed in triplicate for each sample. The small nuclear RNA U6 was used as the normalization control. The miRNA expression level was quantified using the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. Results are presented as the mean ± standard deviation. Statistical analyses were carried out using Stata 8.0 software (Stata, College Station, TX, USA). The comparison of miRNA expression between primary CRC and metastatic carcinomas was carried out using Student's t-test. $P<0.05$ was considered statistically significant. The normalization of data and selection of the differentially expressed miRNAs were performed using GeneSpring GX software.

Results

Clinicopathological characteristics of paired primary and metastatic CRC. Our 4 primary and matched metastatic CRC pairs were all synchronous. Patient characteristics and other clinical findings are shown in Table I. The time from primary tumor to development of brain metastases was ≤3 months. In Case 1 and Case 4 patients, the colorectal primary was identified after the diagnosis of the brain secondary.

miRNA expression profiling in primary CRC versus matched brain metastases. To investigate the role of miRNAs in brain metastasis of human CRC, we conducted a comprehensive miRNA expression profiling of 3 primary CRC and 3 brain-metastatic carcinomas (cases 1,2,3) using the Agilent Human miRNA Microarray V2.0. This microarray contains 15744 probes representing 723 human miRNAs. A total of 19 miRNAs were differentially expressed between primary CRC and brain-metastatic carcinomas. In the brain-metastatic carcinomas, 2 miRNAs were downregulated and 17 miRNAs

Table II. MiRNAs correlated to brain metastasis of human CRC.

miRNA	Regulation in brain-metastatic carcinomas	Chromosome location
miR-199a	Up	1,19,9
miR-133a	Up	20,18
miR-145	Up	5
miR-143	Up	5
miR-10b	Up	2
miR-1	Up	20,18
miR-199a-5p	Up	1,19
miR-145*	Up	5
miR-31	Down	9
miR-125b	Up	21,11
miR-133b	Up	6
miR-22	Up	17
HS_170	Down	0
miR-126*	Up	9
miR-146a	Up	5
miR-28-5p	Up	3
miR-576-5p	Up	4
miR-199b-5p	Up	9
HS_287	Up	0

were upregulated (Table II). These results indicate that these 19 miRNAs may play significant roles in the brain metastasis of human CRC. Additionally, hierarchical cluster analysis based on the expression patterns of these 19 miRNAs accurately separated the primary carcinomas from the metastatic ones (Fig. 1).

Validation of microarray data by real-time RT-PCR. To validate the miRNA microarray results, miR-125b was chosen for analysis by real-time RT-PCR in all 4 CRC cases. The real-time RT-PCR results were in concordance with the miRNA microarray analysis results (Fig. 2). Tissues with higher expression levels of miR-125b were from brain-metastatic lesions.

Discussion

CRC is the most common cancer in China. The common sites of metastasis in CRC include the lung, liver and draining lymph nodes. Brain metastases are relatively rare in CRC. However, the incidence of metastases to the brain is on the increase, due to effective therapies for CRC, which have provided longer survival times, and more effective neuroimaging techniques. The genesis and treatment of brain metastases from CRC has therefore attracted attention (16,17). We also reviewed CRC patient records between 2001 and 2008 in Huashan Hospital. A total of 28 patients who developed brain metastases were identified (18). Our data indicated that the median survival following surgery of brain secondaries was 9.4 months, which is similar to previous reports of patients with brain metastases from CRC in which the median survival was 8.3 months (19).

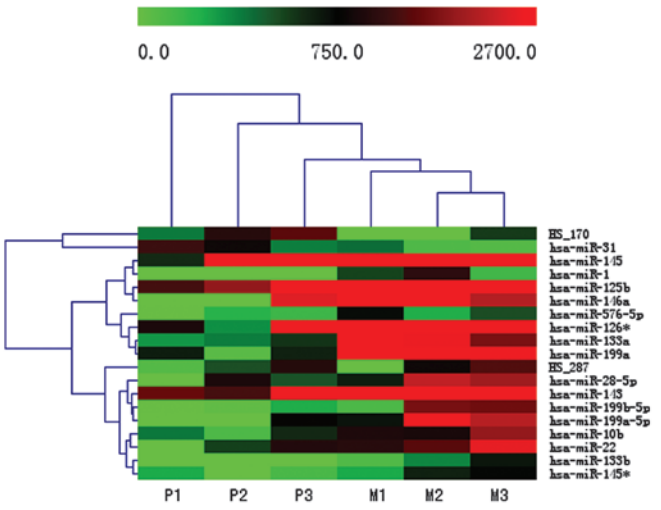

Figure 1. The miRNA expression signature in primary CRC (P) versus the matched brain-metastatic carcinomas (M). The heatmap was based on 19 differentially expressed miRNAs, the expression of which correlated with brain metastasis of human CRC.

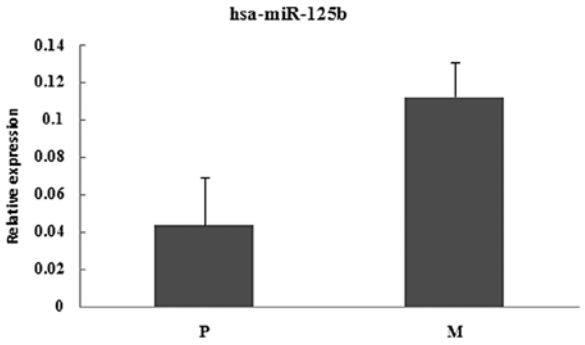

Figure 2. Validation of the differentially expressed miRNAs by real-time RT-PCR. MiR-125b was markedly upregulated in brain metastatic carcinomas (M), but not in primary CRC (P).

It is recommended that a brain scan is performed when lung and/or liver metastases are found, particularly in patients with a rectal or sigmoid colon cancer (4). This finding may lead to earlier diagnosis, amenability to surgical treatment and improved quality of life.

In recent years, advances in molecular biology have markedly expanded our knowledge of the molecular mechanism of metastasis in CRC. Pantaleo *et al* (20) found that the molecular background of liver metastases was different: EGFR and COX-2 were overexpressed in metachronous and synchronous metastases, respectively. These authors proposed that therapies based on EGFR pathway inhibition may be considered for metachronous metastases (such as cetuximab) and therapies based on the inhibition of angiogenesis cross-talking pathways (such as bevacizumab) may be considered for synchronous metastases. However, gene expression profiling of matched CRC and metastases may reveal a small number of key molecular events. Koehler *et al* (5) indicated that a specific expression signature was not identified in matching metastases. Koh *et al* (6) also demonstrated a similar gene expression profile between paired primary and metastatic CRC. These

results are consistent with Bernards and Weinberg's theory that there are no genes or genetic changes that are specifically and exclusively involved in the process of metastasis (7).

It has been established that the potential of a tumor cell to metastasize depends on numerous factors. Mounting evidence has indicated that miRNAs may be key players in the regulation of tumor cell invasion and metastasis (8). miRNAs are small non-coding RNAs that serve as negative regulators of gene expression. Through interactions with the 3' untranslated region of mRNA by partial sequence homology, miRNAs cause gene silencing by mRNA degradation or translation repression. Given the unique feature of their targeting, each single miRNA may have over a hundred targets, and, thus, a large number of protein-coding genes may be regulated by miRNAs (21). Huang *et al* (22) performed a comparison of the miRNA profile of tumor-adjacent non-cancerous colonic samples in CRC with lymph node metastasis with those without lymph node metastasis. Their results indicated that hsa-miR-129* and hsa-miR-137 were significantly differentially expressed. Wang *et al* (11) found that 14 human miRNAs were significantly differentially expressed between colonic tumor tissues and para-cancerous tissues. Using microarray analysis in 3 paired CRCs, Lin *et al* (23) revealed that 28 miRNAs were differentially expressed in CRC with liver metastasis compared with their non-metastatic counterparts.

This study is the first to reveal the miRNA expression profiling of brain metastases from CRC. We selected 3 primary CRCs and 3 brain-metastatic carcinomas for the expression analysis of miRNAs. Our results indicated that an overexpression of miR-145, miR-1, miR-146a, miR-576-5p, miR-126*, HS_287, miR-28-5p, miR-143, miR-199b-5p, miR-199a-5p, miR-10b, miR-22, miR-133b, miR-145*, miR-199a, miR-133a, miR-125b, and a downregulation of miR-31 and HS_170 occurred in brain-metastatic carcinomas. However, previous studies have shown that the expression of miR-143 and miR-145 was downregulated in CRC compared to normal mucosa (24,25). Additionally, miR-31 expression was significantly upregulated in colon tumors relative to hte normal colon tissue (26,27). We propose that a given miRNA may have multiple functions in the processes of carcinogenesis and metastasis.

The quantitative RT-PCR experiments with miR-125b confirmed the expression patterns we found in our microarray experiments. However, Glud *et al* (28,29) proposed that downregulation of miR-125b in malignant melanoma may increase the metastatic ability of this tumor. Liang *et al* (30) found that miR-125b inhibited hepatocellular carcinoma cell migration and invasion through the suppression of oncogene LIN28B expression. We propose that miR-125b may have various functions and target genes in different types of cells. Other miRNAs also regulate miR-125b or its target gene.

Our data are consistent with those of previous studies, which revealed that certain miRNAs are involved in tumor invasion and metastasis, such as miR-199a, miR-10b and miR-31. Song *et al* showed that miR-199a was highly expressed in gastric cancer compared to normal gastric tissues and in metastatic, compared to non-metastatic gastric cancer tissues (31). MiR-199a positively regulated gastric cancer cell proliferation, migration and invasion. Several investigators (32,33) found that miR-10b was highly expressed in metastatic breast cancer cells and positively regulated cell migration and invasion. miR-10b also positively regulated the metastasis of nasopharyngeal carcinoma (34). The expression of miR-31 correlated inversely with metastasis in human breast cancer patients. Loss of miR-31 was associated with cancer progression and metastasis (35-37).

By examining the miRNA expression of primary CRC and brain metastases, we were able to identify miRNAs associated with the brain metastasis of human CRC. Validation of miRNA expression signatures in a larger series is required to improve the understanding of the metastatic process of CRC. Several noteworthy candidate miRNAs may be potential diagnostic markers and therapeutic targets.

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