MicroRNA expression profiles in human colorectal cancers with liver metastases

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Abstract. At present, a full understanding of the mechanisms by which colorectal cancer (CRC) distant metastases form is still beyond our reach because of the intricate regulation of gene expression. MicroRNAs (miRNAs) are shown to be involved in various human diseases including cancers through negative regulation of target gene expression at the post-transcriptional level. However, there are only a few studies on the roles of miRNA aberrations in liver metastasis of human colorectal cancer. To identify miRNA expression patterns associated with liver metastasis in human colorectal cancer, the miRNA expression profiles of colorectal cancer tissues with liver metastasis and their non-metastatic counterparts were studied using microRNA microarrays and further confirmed by quantitative RT-PCR. We show that 28 miRNAs are differentially expressed in the colorectal carcinomas with liver metastasis compared to the non-metastatic counterparts. Of these, 4 miRNAs including miR-150*, miR-125b-2*, miR-1179 and miR-139-3p were up-regulated in colorectal cancers with liver metastasis while the others were down-regulated. The target genes of selected deregulated miRNAs were predicted through bioinformatic techniques with two functional analyses, gene ontology and KEGG analysis, which showed that categories of high enrichment GOs and specific pathways targeted by dysregulated miRNAs were involved in liver metastasis during human colorectum carcinogenesis. Our results indicated that miRNAs are not only involved in carcinogenesis of colorectum, but may also participate in the progression such as with liver metastases in human colorectal cancers.

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Introduction

Colorectal cancer (CRC) is one of the most frequent cancers in the Western world. At present time, CRC has become more and more frequent in China partly attributed to the increasing living standard and transformation of food structure. Several lines of evidence have manifested that distance metastasis particularly shifting to liver was responsible for the poor prognosis in human colorectal cancer patients. Only 10-20% patients with liver metastasis have the chance of surgery. Also, above 50% of the patients suffered recurrence of colorectal cancer after the resection (1). Previous studies have showed that the development and progression of CRC, like others cancers, were results of multiple genetic alterations (2). Nevertheless, a full understanding of the mechanism of CRC distance metastasis is still beyond our reach because of its intricate regulation of gene expression. Few molecules at present could be considered as biomarkers which could early differentiate colorectal cancers with distance mestastasis from their early stage counterparts. Fortunately, identication of multiple microRNAs (miRNAs) along with a comprehensive description of the miRNA/mRNA interaction network may add a new level to our knowledge of the gene regulation in the liver metastasis in human colorectal cancer.

MicroRNAs, a new class of non-coding RNAs, were discovered recently and shown to be involved in various human diseases including cancers through negatively regulating the target gene expression in post-transcriptional level (3). Cleaved from 70 to 100 nt hairpin pre-miRNA precursors, which were generated from primary transcript miRNAs, miRNAs were then modified by cytoplasmic RNase III Dicer into 22 nt miRNA duplex: one strand of the short-lived duplex is degraded, whereas the other strand, which serves as mature miRNA, is incorporated into the RNA-induced silencing complex (RISC) and drives the selection of target mRNAs containing antisense sequences in 3'-untranslated region (4). According to the current views,

Table I. Clinicopathological data in 6 colorectal cancers in microRNA microarray.

Case	1	2	3	4	5	6
Age	71	55	43	81	77	43
Gender	Male	Female	Female	Female	Female	Female
Tumor size (cm)	5x4	8x8	5x5	10x10	10x10	3x3
Depth[a]	Serosa	Mucosa	Serosa	Serosa	Serosa	Serosa
Histological type	Moderate	Moderate	Moderate	Poor	Moderate	Poor
Lymphatic invasion	(-)	(-)	(-)	(+)	(+)	(+)
Liver metastasis	(-)	(-)	(-)	(+)	(+)	(+)
Clinical stage[b]	IIA	I	IIA	IV	IV	IV

[a]Depth indicate the invation range of the tumor (serosa, tumor invated to seroma, and mucosa, tumor invated to mucosa). [b]TNM stage system formulated by AJCC/NCCN (2009) according to the tumor, node and metastasis was used in these patients.

imperfect complementarity leads to repression of translation of the target mRNA and is the main mechanism of miRNA regulation in animals, whereas perfect sequence homology induces degradation of the target mRNA and is mainly detected in plants (5). Previous studies have shown that miRNAs involved in cancer development and progression are oncogenes or tumor suppressor genes (6-11). Moreover, a number of studies foused on the significance of miRNAs in human colorectal cancer have been reported (12-15). However, few studies related to the roles of aberration of miRNAs in liver metastasis of human colorectal cancer have been reported.

Recognition of miRNAs that are differentially expressed between relatively early stage cancers and advanced malignant tumors may help to identify those that are involved in human cancer progression and establish the basis to unravel their pathogenic role. In this study, we present the results of genome wide miRNA expression profile of a set of colorectal carcinomas with and without liver metastasis which could reveal the specific miRNAs correlated with colorectal cancer metastasis. The differentially expressed miRNAs were selected, validated and subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The miRNAs and target genes predominating in the miRNA gene regulatory networks were further investigated in CRC in an attempt to provide new insight into the understanding of the biological process of CRC liver metastasis.

Materials and methods

Patients and specimen. With the approval of the institutional review board of the first hospital affiliated to Soochow University, 25 colorectal cancer tissue samples were obtained from 5 patients with liver metastasis and 20 patients without distance metastasis who underwent colorectum resection on January 2009 through May 2009. None of the patients received preoperative treatments, such as radiation and/or chemotherapy. All tissue samples were collected and immediately snap-frozen in liquid nitrogen, and stored at -80˚C until RNA extraction. Exclusion criteria were a previous history of local or systemic treatment for CRC. Written informed consent from each patient was obtained according to the institutional regulations.

RNA extraction and miRNA array hybridization. Total RNAs from 6 human colorectal cancers patients with or without liver metastasis were analyzed by microRNA microarray. The clinicopathological characteristics of colorectal cancer patients included in microarray analysis are described in Table I. Total RNA isolation was done with TRIzol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Concentration and purity of the total RNAs were assessed by a spectrophotometer and RNA integrity was verified using an Eppendorf Biophotometer (Eppendorf, Germany). RNA labeling and hybridization on miRNA micro-array chips were done according to the microRNA expression profiling assay guide (Illumina, USA). Briefly, total RNA (200 ng) was polyadenylated with a PAP enzyme (Poly-A Polymerase), then converted to cDNA using a biotinylated oligo-dT primer with a universal PCR sequence at its 5-nd. The miRNA expression was targeted by sequences with chimeric oligos containing universal PCR amplification primer sites. One miRNA-specific oligo (MSO) was used to assay each miRNA. Then the cDNA templates were hybridized with the set of MSOs that corresponds to all the targeted miRNAs. After the oligo annealing, mis-hybridized and non-hybridized oligos were washed away. A polymerase was then added, causing the MSOs to undergo extension (i.e., second-strand cDNA synthesis). The MSOs were extended only if their 3 bases are complementary to the cognate sequence in the cDNA template. The cDNA templates were all amplified with a pair of common PCR primers. The primer on the strand that was complementary to the array was fluorescently labelled. After PCR amplification, the labelled, single-stranded product were hybridized to Illumina human miRNA microarrays, which contain probes for 1146 human miRNAs representing a majority of known miRNAs (16) plus additional novel content derived using Illumina sequencing technology for 14 h at 45˚C. The microarrays were then washed using Illumina-prepared buffers. The Illumina iScan

System (Illumina) was then used to measure the fluorescence intensity at each addressed bead location. The intensity of the signal corresponds to the quantity of the respective miRNA in the original sample. Normalization was performed using the average normalization method.

Computational analysis of miRNA microarray data. The intensity of each hybridization signal was evaluated using Feature Extraction Software. Feature Extraction analysis examined multiple probes and multiple features per probe and studied the measurements and errors for each miRNA. Average values of the replicate spots of each miRNA were background subtracted, normalized, and subjected to further analysis. Class comparison analysis using t-tests identified miRNAs that were differentially expressed in colorectal cancers with liver metastasis and their non-metastasis counterparts.

Quantitative real-time polymerase chain reaction. MiR-139-3p, miR-19a, and RNU6B (internal control) specific cDNAs synthesis were carried out with the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. A quantitative PCR was performed using miScript SYBR-Green PCR Kit (Qiagen). The reactions were incubated in a 96-well optical plate at 95˚C for 15 min followed by 40 cycles of 5 sec at 95˚C, 31 sec at 61˚C and then were held at 4˚C. Expression analysis was performed in triplicate for each sample. Both melting curve analysis and agarose gel run were used to confirm the specificity of the amplification reactions. The small nuclear RNA U6 was used as the normalization control. The miRNA expression level was quantified using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, with the ratio of the median expression sample among all cancers with liver metastasis/all non-metastasis samples being used as the calibrator.

miRNA target prediction and geneontology (GO) analysis. According to the bioinformatic techniques, target genes of deregulated miRNAs in advanced colorectal cancers with liver metastasis compared with their non-metastasis counterparts were picked out based on the Sanger MicroRNA Bank. In addition, these target genes were narrowed down according to the Unigene data base which is specific to enterology. GO analysis was applied in order to organize genes into hierarchical categories and uncover the miR-Gene Regulatory Network on the basis of biological process and molecular function (17). Two-sided Fisher's exact test and χ^2 test were used to classify the GO category, and the false discovery rate (FDR) was calculated to correct the P-value. We chose only GOs that had a P-value of <0.001 and a FDR of <0.05. Within the signicant category, the enrichment Re was given by: $Re = (n_f/n)/(N_f/N)$ where n_f is the number of flagged genes within the particular category, n is the total number of genes within the same category, N_f is the number of flagged genes in the entire microarray, and N is the total number of genes in the microarray.

KEGG pathway annotation based on miRNA expression profile. The aberrant miRNA targets were collected, and subjected to KEGG pathway annotation using the david gene annotation tool (http://david.abcc.ncifcrf. gov/) (18). A two-sided Fisher's exact test and χ^2 test were used to classify the enrichment (Re) of pathway category, and the false discovery rate (FDR) was calculated to correct the P-value. Within a KO, the enrichment (Re) was given by where n_f and n represent the number of target genes and total genes, respectively, in the particular KO, and N_f and N represent the number of genes among the entire differential miRNA-corresponding target genes and the total number of genes on the pathway, respectively. We chose only pathways that had a P-value of <0.001 and an FDR of <0.05. The regulator pathway annotation was also performed on the basis of scoring and visualization of the pathways collected in the KEGG database (http://www.genome.jp/kegg/).

Statistical analysis. All the results are expressed as the means ± standard deviation (SD). Statistical analysis was done with Student's t-test for comparison of two groups in microarray analysis in which differences with P<0.001 were considered statistically signicant, and ANOVA for comparisons of expression of selected aberrant miRNAs in quantitative RT-PCR assay. In real-time PCR analysis, miRNAs were designated as overexpressed if expression was >2.0-fold and as underexpressed if expression was <0.5-fold compared to 20 non-metastasis cancers in all 5 clinical colorectal cancer samples with liver metastasis while difference was considered significant at P<0.05, performed using SPSS software 16.0 (SPSS, Chicago, IL, USA).

Results

Differentially expressed miRNAs in colorectal cancers with and without liver metastasis. To investigate the differential expression of miRNAs in human colorectal cancers with and without liver metastasis, array-based miRNA profiling of human colorectal cancer was performed. Out of 1146 human miRNAs assayed, 4 miRNAs including miR-150*, miR-125b-2*, miR-1179 and miR-139-3p were identified that had higher expression levels in colorectal cancer tissues with liver metastasis than in non-metastasis counterparts while the other 24 miRNAs were down-regulated (Fig. 1A, displaying 25 mature miRNAs covered in miBase 12.0). Of which, miR-150* was the most up-regulated miRNA while miR-18b was the most underexpressed in the colorectal cancer tissues with liver metastasis analyzed.

Validation of microarray data by miRNA real-time RT-PCR analysis. To confirm the microarray findings, we measured the expression levels of miR-139-3p and miR-19a, the novel miRNAs of particular interest in colorectal cancer in the 3 paired specimens that were used in the microarray study described above, using quantitative RT-PCR (qRT-PCR) in all 25 colorectal cancer cases. As expected, a statistically significant up-regulation in the expression of miR-139-3p was observed in liver metastasis group compared with the non-metastasis group (mestastasis group, 1.75±0.40 vs. non-metastasis group, 0.69±0.58, P<0.05) while miR-19a was down-regulated (mestastasis group 0.39±0.20 vs. non-metastasis group, 1.38±0.98. P<0.05) (Fig. 1B). The quanti-

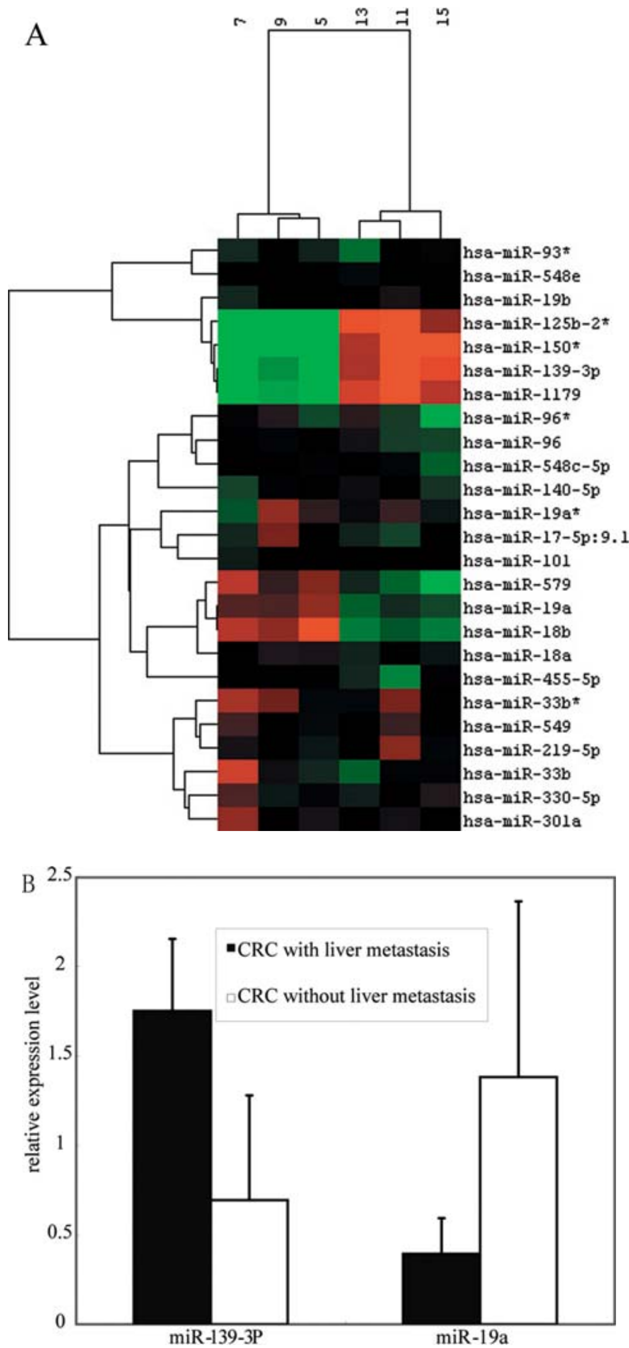

Figure 1. (A) miRNA profiles differentiate colorectal cancers with liver metastasis (cases 11, 13 and 15) from their non-metastasis counterparts (cases 5, 7 and 9). Samples consist of colorectal cancers from six patients. Both down-regulated and up-regulated miRNAs were identified in CRCs with liver metastasis (P<0.001) (x500). (B) The expression levels of miR-139-3p and miR-19a were detected by using quantitative RT-PCR (qRT-PCR) in all 25 colorectal cancers. MiR-139-3p was found up-regulated while miR-19a was down-regulated in CRCs with liver metastasis compared with the non-metastasis cases (P<0.05) (x600).

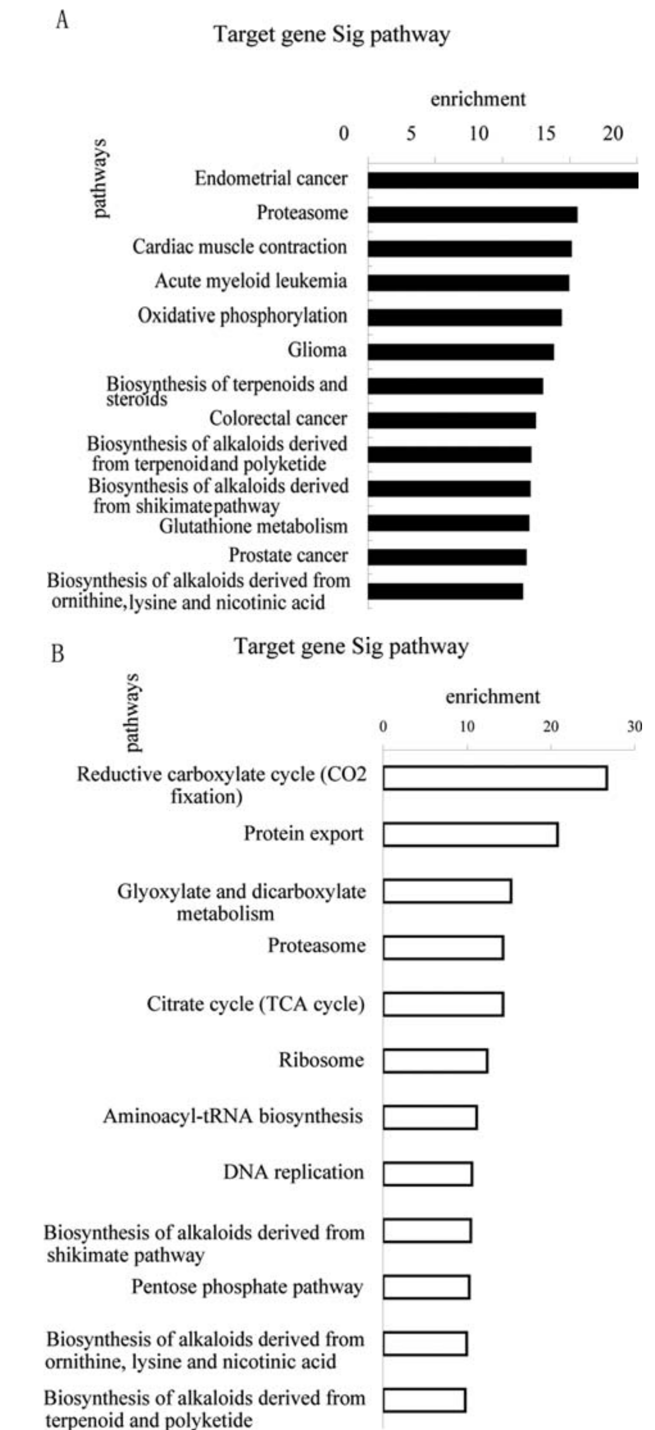

Figure 2. Pathway analysis based on miRNA targeted genes. (A and B) Significant pathways targeted by up-regulated and down-regulated miRNA, respectively. The vertical axis is the pathway category, and the horizontal axis is the enrichment of pathways. (A) Pathways probably up-regulated by enrichment miRNAs in CRCs with liver metastasis (original magnification, x600). (B) Pathways probably down-regulated by enrichment miRNAs in CRCs with liver metastasis (original magnification, x600).

tative RT-PCR results were in concordance with microarray analysis results.

Bioinformatics interpretation revealed that the GOs and signaling pathways are regulated by miRNAs. GOs were signicantly regulated by miRNAs, the P-value and FDR were <0.001 and <0.05, respectively. The high-enrichment GOs targeted by overexpressed miRNAs were carbohydrate and lipid metabolism, cell proliferation, regulation of apoptosis and cell cycle, cell differentiation, and regulation of cell adhesion. Similarly, signicant GOs corresponding to underexpressed miRNAs appeared to be metabolism, apoptosis, cell cycle, differentiation, and DNA repair. Significant GOs according to the enrichment are listed in Table II. Another functional analysis of miRNAs by KEGG

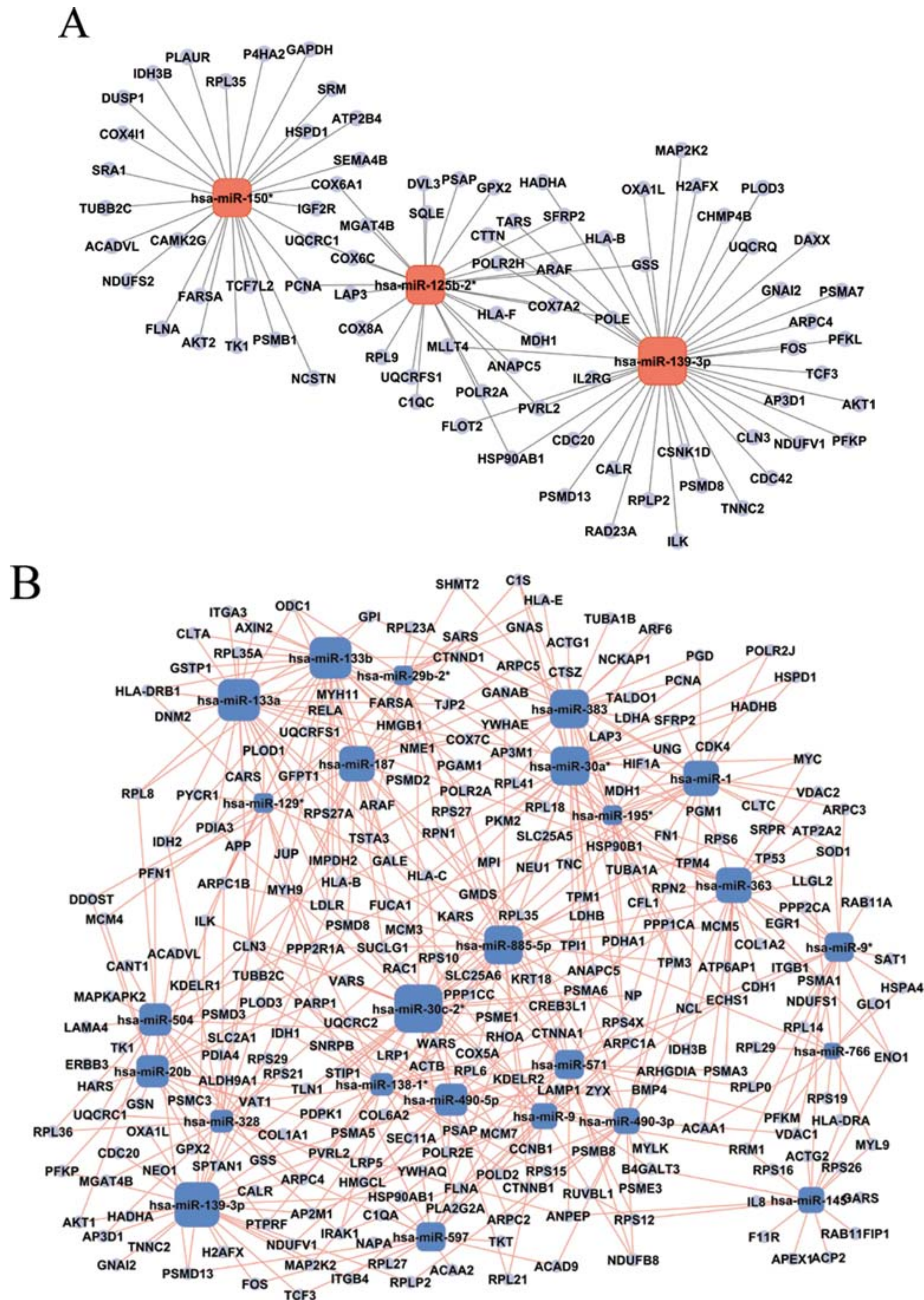

Figure 3. MicroRNA-Gene network. Red and blue box nodes represent miRNAs which are up-regulated and down-regulate respectively, and grey cycle nodes represent mRNA. Edges show the inhibitory effect of microRNA on mRNA. Up-regulated and down-regulated microRNA have separate, specific targets. The upper subgraph (A) shows overexpression microRNA-mRNA network and the lower subgraph (B) is the underexpression microRNA-mRNA network. Three underexpressed miRNAs (miR-548c-5p, miR-33b, and miR-19a) showed the most target mRNAs with degree above 39. In contrast, miR-139-3p was the highest degree in overexpressed miRNAs. (Original magnification, x300).

showed that 25 signaling pathways were up-regulated and 50 pathways were down-regulated in colorectal cancers with liver metastasis involving a variety of cellular processes such as metablotic (glutathione, purine, glycolysis/gluconeogenesis, pyruvate, pyrimidine), oncogenic (colorectal cancer, prostate cancer renal cell carcinoma, and endometrial cancer), signal transduction (ErbB signaling pathway, insulin signaling pathway, Wnt signaling pathway, calcium signaling pathway, PPAR signaling pathway) as well as adhesive (tight junction, adherens junction, focal adhesion, ECM-receptor interaction) pathways. Similarly, top 50% up-regulated pathways and 25% down-regulated signal pathways selected according to the enrichments are listed in Fig. 2. Furthermore, miRNA-gene network of overlapping target genes selected in GOs and pathway analysis and their differential miRNAs were performed successfully (Fig. 3A and B). The major aberrant miRNAs such as miR-548c-5p, miR-139-3p, miR-33b and miR-19a and the major target genes including

Table II. MicroRNA targets of significant GO in colorectal cancers with liver metastasis.

GO ID	GO name	P-value	FDR	Enrichment
Up-regulated GOs by GO analysis[a]				
GO:0045884	Regulation of survival gene product expression	0.00027	0.00129	99.05392
GO:0010466	Negative regulation of peptidase activity	0.00054	0.00188	74.29044
GO:0034614	Cellular response to reactive oxygen species	2.1E-05	0.00026	63.67752
GO:0006665	Sphingolipid metabolic process	8.4E-05	0.00057	20.49391
GO:0019538	Protein metabolic process	0.00012	0.00076	18.57261
GO:0007163	Establishment or maintenance of cell polarity	0.0004	0.00162	13.82148
GO:0030182	Neuron differentiation	6.4E-05	0.00048	13.75749
GO:0022900	Electron transport chain	0.00014	0.00081	11.79213
GO:0051436	Negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	3.5E-05	0.00034	11.14357
GO:0031145	Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein ocatabolic process	3.7E-05	0.00036	11.00599
GO:0051437	Positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	4.9E-05	0.0004	10.48806
GO:0006096	Glycolysis	0.00033	0.00146	9.775058
GO:0042981	Regulation of apoptosis	2E-07	4.4E-06	9.711169
GO:0006091	Generation of precursor metabolites and energy	0.00077	0.00222	8.163785
GO:0006979	Response to oxidative stress	0.0004	0.00162	7.189398
GO:0007050	Cell cycle arrest	0.00061	0.00201	6.652875
GO:0030036	Actin cytoskeleton organization	0.00043	0.00168	5.843068
GO:0006464	Protein modification process	4.2E-06	7.5E-05	5.571783
GO:0055114	Oxidation reduction	4.1E-10	3.7E-08	4.707886
GO:0051301	Cell division	0.00019	0.00103	4.475328
GO:0044419	Interspecies interaction between organisms	5E-05	0.0004	4.359341
GO:0008380	RNA splicing	0.00076	0.00221	3.761541
GO:0006468	Protein amino acid phosphorylation	0.00051	0.00184	2.898197
GO:0030154	Cell differentiation	0.00087	0.00239	2.88107
Down regulated GOs by GO analysis[b]				
GO:0006102	Isocitrate metabolic process	0.00014	0.0009	28.56786
GO:0035162	Embryonic hemopoiesis	0.00035	0.00179	22.85429
GO:0031529	Ruffle organization	3.1E-05	0.00027	21.76599
GO:0042273	Ribosomal large subunit biogenesis	0.00068	0.00306	19.04524
GO:0051881	Regulation of mitochondrial membrane potential	0.00068	0.00306	19.04524
GO:0042176	Regulation of protein catabolic process	9.5E-07	1.2E-05	17.58022
GO:0008333	Endosome to lysosome transport	0.00017	0.00104	15.23619
GO:0006937	Regulation of muscle contraction	4.1E-06	4.1E-05	14.28393
GO:0006613	Cotranslational protein targeting to membrane	0.00027	0.00147	13.85108
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	0.00027	0.00147	13.85108
GO:0006099	Tricarboxylic acid cycle	8.8E-10	1.8E-08	12.69683
GO:0018279	Protein amino acid N-linked glycosylation via asparagine	9.1E-06	8.7E-05	12.69683
GO:0007052	Mitotic spindle organization	0.0004	0.00198	12.69683
GO:0042542	Response to hydrogen peroxide	0.0004	0.00198	12.69683
GO:0044262	Cellular carbohydrate metabolic process	0.0004	0.00198	12.69683
GO:0009306	Protein secretion	1.8E-05	0.00016	11.42714
GO:0051436	Negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	1.1E-17	6.5E-16	10.95101

Table II. Continued.

GO ID	GO name	P-value	FDR	Enrichment
GO:0031145	Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	1.6E-17	7.9E-16	10.81582
GO:0051437	Positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	3.5E-18	2.5E-16	10.75496
GO:0045785	Positive regulation of cell adhesion	0.00021	0.00122	10.02381
GO:0010033	Response to organic substance	0.00035	0.00182	9.069162
GO:0000059	Protein import into nucleus, docking	9.5E-05	0.00068	8.790111
GO:0006268	DNA unwinding during replication	9.5E-05	0.00068	8.790111
GO:0015986	ATP synthesis coupled proton transport	9.5E-05	0.00068	8.790111
GO:0043193	Positive regulation of gene-specific transcription	0.00045	0.00219	8.656927
GO:0044267	Cellular protein metabolic process	0.00012	0.0008	8.464551
GO:0006635	Fatty acid ß-oxidation	0.00085	0.00362	7.618096
GO:0006956	Complement activation	0.00085	0.00362	7.618096
GO:0008624	Induction of apoptosis by extracellular signals	3.4E-05	0.00029	6.925542
GO:0006418	tRNA aminoacylation for protein translation	9.8E-07	1.2E-05	6.868775
GO:0006958	Complement activation, classical pathway	3.6E-06	3.6E-05	6.801872
GO:0006820	Anion transport	0.00046	0.00224	6.72185
GO:0008652	Amino acid biosynthetic process	0.00015	0.00092	6.665834
GO:0006096	Glycolysis	1.7E-07	2.4E-06	6.515477
GO:0042060	Wound healing	0.00064	0.00292	6.348413
GO:0006986	Response to unfolded protein	2.8E-07	3.6E-06	6.268054
GO:0000398	Nuclear mRNA splicing, via spliceosome	1.2E-16	5.3E-15	6.192049
GO:0006270	DNA replication initiation	0.00087	0.00368	6.014286
GO:0006606	Protein import into nucleus	0.00087	0.00368	6.014286
GO:0045454	Cell redox homeostasis	8.4E-08	1.2E-06	5.830176
GO:0006898	Receptor-mediated endocytosis	0.00042	0.00209	5.67305
GO:0015992	Proton transport	2.7E-06	2.9E-05	5.643034
GO:0043065	Positive regulation of apoptosis	2.7E-06	2.9E-05	5.643034
GO:0007584	Response to nutrient	7.6E-05	0.00057	5.441497
GO:0042981	Regulation of apoptosis	1.4E-09	2.7E-08	5.228105
GO:0051260	Protein homooligomerization	0.00071	0.00317	5.228105
GO:0006414	Translational elongation	4E-15	1.4E-13	5.18742
GO:0030521	Androgen receptor signaling pathway	0.0003	0.0016	5.164811
GO:0006094	Gluconeogenesis	0.00081	0.00348	5.127565
GO:0016049	Cell growth	0.00091	0.0038	5.030818

[a]GOs targeted by up-regulated miRNA. [b]GOs targeted by down-regulated miRNA. These GOs show increased enrichment, P-values and FDRs.

CCNL1, SRA1, PSAP, SREBF1, PSMB1, PFKP, HSPA8, HSP90AB1, HADHA as well as CTSK are outlined according to the network.

Discussion

Aberrant miRNA expression patterns have been described in a variety of hematologic and solid organ malignancies including gastrointestinal cancers. MiRNA, one of the regulators of gene expression, was found involved in the tumorigenesis of colorectal cancer cells acting as oncogene or tumor suppressor genes (12-15). However, less studies exist related with miRNA expression accounting for the distance metastasis of the colorectal cancer. Therefore, in this study, expression profiling of 1146 miRNAs based on illumina miRNAs array platform in clinical samples of colorectal cancer with or without liver metastasis was carried out to identify advanced colorectal cancer signatures and to use miRNA expression profiles in classifying colorectal cancer with liver metastasis. The confirmation of the array data was done by using quantitative RT-PCR.

The results, by microarray analysis in 3 paired colorectal cancers, showed 28 miRNAs were dysregulated in colorectal cancers with liver metastasis compared with their non-metastasis counterparts. Among these differentially expressed miRNAs, miR-150*, miR-125b-2*, miR-139-3p

and miR-1179 demonstrated overexpression in colorectal cancer with liver metastasis, whileas the other 24 miRNAs were underexpressed. In addition, up-regulation of miR-139-3p and down-regulation of miR-19a in colorectal cancer with liver metastasis were detected by the quantitative RT-PCR asssay, which further confirmed the microarray results. The set of 28 miRNAs might predict the nature of samples analyzed in human colorectal cancers with liver metastasis. These results showed little doubt that aberrant expression of miRNAs might be involving in liver metastasis of human colorectal cancer and to be further related to the outcome of colorectal cancer patients.

Alteration of miR-125b-2*, miR-301a, miR-219-5p, miR-549, miR-455-5p as well as miR-33b, acting as cancer associated genes, have been found involved in several cancer types such as myeloma, bladder and prostate cancer and endometrial serous adenocarcinomas (19-22). Our microarray profilings demonstrated that these five miRNAs were also dysregulated in colorectal cancer samples with liver metastasis compared with the paired tissues without liver metastasis, which might further imply their regulating role in pathogenesis of distance metastasis in human colorectal cancer. In addition, Strillacci *et al* (23) reported the down-regulation of miR-101 in colon cancer cell lines and the inverse correlation between COX-2 and miR-101 expression. This correlation was supported by data collected *ex vivo*, in which colon cancer tissues and liver metastases derived from CRC patients were analyzed. Similarly, miR-101 was underexpressed in our colorectal cancer samples with liver metastasis in comparison to their relatively early patients without metastasis, which confirmed the previous study. Consequently, aberration of miR-101 might play a crucial role in distance metastasis of human colorectal cancer, which provided a novel target to be further studied in the future. Previous studies assumed that miR-17-92 cluster might manifest differrent roles in respected organs, oncogene or tumor suppressor, according to the targeted genes (24,25). Diosdado *et al* (26) found that dysregulation of miR-17-92 cluster involved in carcinogenesis of colorectum, showed their oncogene role. However, the role of expression of miR-17-92 cluster during liver metastasis in colorectal cancer was largely unknown. In this study, miR-19a, miR-19b, miR-18a and miR-18b, mostly cleavage products of miR-17-92 cluster, were detected deregulated in human colorectal cancer with liver metastasis, partly indicating their pathogenetic role in liver metastasis in colo-rectal cancer. In addition, several miRNAs, including miR-150*, miR-1179, miR-139-3p, miR-140-5p, miR-93*, miR-548e, miR-19a*, miR-330-5p, miR-548c-5p, miR-33b*, miR-17-5p:9.1 as well as miR-579, which were not previously detected associated with any cancers were found dysregulated in colorectal cancer with liver metastasis in our study, suggesting that the alteration of these miRNAs might also participate in the progression of human colorectal cancer.

At present, lack of comprehensive knowledge on the targets of the miRNAs is responsible for poor understanding on the biological functions of the aberrant miRNAs in development and progression of human colorectal cancer. To partially overcome this limitation, in this study we further predict the potential target genes of the alterative miRNAs in colorectal cancer with liver metastasis by using presently available computational approaches. Through the bioinformatic bank, several target genes which involve several cancer associated genes were found specific to differentially expressed miRNAs. Consistent with previous studies, computer and bioinformatic based predictions in our study showed that many mRNAs could be regulated by miRNA and every RNA simultaneously been manipulated by several miRNAs, which indicated the complicated regulation network between the miRNAs and their target mRNAs. Despite these complex characteristics, gene ontology enrichment analysis, which was based on the reported and predicted target genes of these aberrant miRNAs, was developed to shed light on whether particular functions are enriched among genes controlling distinctive characteristics between reference group and test group. Abundant high-enrichment GOs of target genes regulated by differential miRNAs might involve several biological processes such as carbohydrate and lipid metabolism, cell proliferation, regulation of apoptosis and cell cycle, cell differentiation, and regulation of cell adhesion. According to the KEGG analysis which in accordance with the GO assay, specific pathways targeted by deregulation miRNAs were found involved in the progression of liver metastasis in human corectal cancer. Changes in miRNAs revealed that 26 pathways were up-regulated and 50 pathways were down-regulated during liver metastasis. Of which, important metablotic (glutathione, purine, glycolysis/gluconeogenesis, pyruvate, pyrimidine), oncogenic (colorectal cancer, prostate cancer renal cell carcinoma, and endometrial cancer), signal transduction (ErbB signaling pathway, insulin signaling pathway, Wnt signaling pathway, calcium signaling pathway, PPAR signaling pathway), adhesive (tight junction, adherens junction, focal adhesion, ECM-receptor interaction) signal pathways were abundant among the significantly enriched ones. Most of them have already been reported to participate in carcinogenesis and invasion or even metastasis of the colorectum. For instance, mutations in the Adenomatous Polyposis Coli (APC) gene (GenBank accession no. 324) are typically the critical initiating event in cases of Familial Adenomatous Polyposis (FAP)-associated as well as most sporadic cancers resulting in activation of the Wnt signaling pathway (2). Both insulin and IGF-1 can signal through their receptors to promote cellular proliferation and angiogenesis, or inhibit apoptosis in CRC cells (27-29). This functional identity revealed by different bioinformatic interpretation confirmed that miRNAs have regulatory effects on liver metastasis in human colorectal cancer by affecting several signaling pathways.

Furthermore, miRNA-gene network of overlapping target genes selected in GOs and pathway analysis integrated the bioinformatic findings and then outlined the major aberrant miRNAs such as miR-548c-5p, miR-139-3p, miR-33b and hsa-miR-19a and the major target genes including CCNL1, SRA1, PSAP, SREBF1, PSMB1, PFKP, HSPA8, HSP90AB1, HADHA as well as CTSK. However, we have to point out that the results acquired through bioinformatic approach will first need to be validated in a much larger cohort of colon cancer patients in future studies. At the same time, it should be noted that our results acquired in this study were based on

relatively few clinical samples. However, despite its preliminary character, this study clearly indicated the role of miRNAs in liver metastasis of human colorectal cancer.

Collectively, our results suggested that not only are miRNAs involved in carcinogenesis of colorectum, but might also participate in the progression of liver metastasis in human colorectal cancers, regulating post-transcriptionally their target genes through several pathways and then affecting transformation of colon cells and distance metastasis. The ability of miRNA to affect multiple genes in various pathways makes them a logical target for the investigation of novel anti-tumoral therapies. However, the distinct role of these aberrant miRNAs need further validation in future research including more clinical cases.

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