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miRNA expression profile of colon cancer stem cells compared to non-stem cells using the SW1116 cell line

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Abstract. Colorectal cancer (CRC) is one of the major causes of cancer-related mortality worldwide. Recent studies revealed that there is a relationship between CRC occurrence and microRNA (miRNA) function. Stem cells are a type of cells that have the ability to self-renew and to proliferate extensively while maintaining the undifferentiated state. Cancer stem cells (CSCs) are closely linked to tumor recurrence and metastasis. To this end, we evaluated the miRNA expression differences between colon CSCs and non-stem cells using the SW1116 cell line, to determine the relationship between tumor stem cells and tumor biological behavior. We isolated populations of colon CSCs with the $CD133^+/CD44^+$ and $CD133^-/CD44^-$ surface phenotype from a human SW1116 colon adenocarcinoma cell line using flow cytometry. The expression of miRNA and mRNA of both sets of cells was examined with miRNA and mRNA arrays. Bioinformatic methods were used to analyze microarray results. We completed gene ontology analysis, pathway analysis, miRNA target gene prediction with databases. We identified a colon stem cell miRNA expression profile comprising 31 upregulated and 31 downregulated miRNAs, such as miR29a, miR29b, miR449b and miR4524. Some of these differentially expressed miRNAs may be involved in the regulation of stem cell differentiation. Gene ontology and pathway analyses showed that the differences are closely related to the function of the cell cycle, cell differentiation, signaling pathway, cytoskeletal proteins and cell-matrix adhesion in colon cancer stem cells. We found that miRNAs play an important role in regulating the expression of colon CSC characteristics. By regulating the expression of CSC signaling pathways, cytoskeleton and membrane proteins, miRNAs give tumor stem cells the macrobiological behavior of recurrence and metastasis. This study provides a new perspective on CRC metastasis and recurrence.

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide (1) and has created new challenges to the methods and tools of treatment. CRC is often the result of a combination of environmental and genetic mutations, accompanied by a variety of gene expression profile changes (2-4). Genetic mutations, including point mutations, chromosomal translocation and gene amplification, result in oncogenes and tumor suppressor gene mutations involved in cancer development (2). Tumor cells are parts of tissues that have lost normal regulation of their growth, resulting in clonal dysplasia and the formation of neoplasm, following the impact of a variety of carcinogenic factors. Inappropriate expression of tumor-suppressor genes or oncogenes is regarded the principal cause of tumorigenesis.

The biological behavior of cancer, including carcinogenesis and functional heterogeneity, can be explained by the cancer stem cell (CSC) hypothesis. According to this model, CSCs, which exhibit stem-like features, are involved in tumor formation, proliferation, differentiation, metastasis and resistance to therapy (5-7). Through their ability of self-renewal and unlimited proliferation, tumor stem cells maintain the vitality of the tumor cell population, and through their increased movement and migration, tumor stem cells make cell metastasis possible. With a variety of drug-resistant molecules, tumor stem cells allow the tumor to become non-sensitive to external physical and chemical factors. At the same time, numerous studies have indicated that some cytokines, protein-coding genes and its products are involved in the maintenance of the biological characteristics of CSCs (8-10). However, the underlying mechanisms remain largely unknown.

microRNAs (miRNAs), approximately 18-24 nt in length, are a class of non-coding single-stranded RNA molecules in eukaryotes. miRNAs regulate their target gene expression through post-transcriptional regulation, which includes mediating degradation of target mRNA, inhibiting target mRNA translation through complementary combining with the target mRNA 3'-untranslated region (UTR) completely/incompletely

(11-14). Similar to other transcription factors, miRNAs are adjustment factors which can determine cell fate. miRNAs and other post-transcriptional regulatory mechanisms are regarded the mechanisms that control gene expression (11,15,16).

In recent years, the combination of computer science, information technology, mathematical theory and gene chip technology, has given rise to an interdisciplinary science, bioinformatics. Bioinformatics includes biological data handling, processing of the genetic and physical map, nucleotide and amino acid sequence analysis, the discovery of new genes and protein structure prediction. With bioinformatics we can increase our knowledge of genes, and from our previous understanding of a single gene, we can now examine the genes in the whole genome level organizational structure and information structure, and examine the mutual relationships between gene location, structure and function.

However, the reasons underlying the generation, development, recurrence and metastasis of CRC remain unclear and the role of stem cells in the tumor biological processes has yet to be fully elucidated. Using a combination of bio-chip and computer bioinformatics, herein we report a comprehensive analysis of stem cell properties in the CRC cell line.

Materials and methods

Cell culture and tissues. The SW1116 human CRC cell line was obtained from the Shanghai Chinese Academy of Sciences (CAS). The CRC cells were carefully cultured in RPMI-1640 medium containing 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were cultured at 37˚C, in a 5% CO_2 atmosphere with 95% humidity.

We separated $CD133^+CD44^+$-positive and $CD133^-CD44^-$-negative cells using flow cytometry. $CD133^+CD44^+$-positive cells were cultured in serum-free DMEM/F12 with EGF (10 ng/ml) and βEGF (10 ng/ml). $CD133^-CD44^-$-negative cells were cultured in DMEM/F12 medium with fetal calf serum. Cells were cultured at 37˚C, in a 5% CO_2 atmosphere with 95% humidity (Fig. 1).

Extraction of total cellular RNA. Total-RNA extraction was performed with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted RNA samples were quantified by NanoDrop 1000 (Nanodrop, Wilmington, DE, USA). To remove any genomic DNA contamination, the samples were treated by DNase (DNA-free kit; Ambion, Austin, TX, USA).

miRNA chip analysis. The samples were analyzed with Human microRNA OneArray® v3 which is produced by Phalanx Biotech Group (Belmont, CA, USA). Human microRNA OneArray microarrays are made of polydeoxynucleotide probes spotted onto a proprietary chemical layer coated on top of a 1"x3" (25x75 mm) standard format microarray glass slide. Each probe is spotted onto the array in a highly consistent manner using proprietary, non-contact spotting technology. Each microarray contains 1711 unique human miRNA probes and 189 experimental control probes. Each unique probe has 3 features, and probes contain 100% of Sanger miRBase v17 miRNA content. We used ULS miRNA labeling kit (Kreatech, Durham, NC, USA) to label target. We used miRNA OneArray Hyb Buffer V3 and miRNA OneArray Hybridization Buffer II to complete the hybridization process. All these steps were conducted in accordance with the manufacturer's instructions. We used an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) to scan miRNA chip, and GenePix 4.1 data analysis software.

mRNA chip analysis. We analyzed samples with Illumina® Whole-Genome Gene Expression Direct Hybridization Assay system (direct hybridization assay) which integrates Illumina proprietary BeadArray technology, a precise microarray scanning system (the Illumina HiScan™ or iScan System or the Illumina BeadArray™ Reader), hybridization equipment and accessories, and standard, off-the-shelf sample labeling protocols. First we amplified RNA samples with Illumina® TotalPrep RNA Amplification kit. The procedure consists of reverse transcription to synthesize first strand cDNA, second strand cDNA synthesis, cDNA purification, *in vitro* transcription to synthesize cRNA and cRNA purification. All steps followed the instruction manual. The BeadArray chip analysis and data analysis were performed by YiKe Co., Shanghai, China. All analyses were conducted in accordance with the manual.

Real-time polymerase chain reaction assay for miRNA. Total-RNA was extracted from cells with TRIzol (Invitrogen). Total-RNA was assessed by measuring the absorbance at 260 nm. cDNA was synthesized using ImProm-II reverse transcriptase. With strand cDNA (0.5 μl), forward and reverse primers (both 0.5 μl) and SYBR green supermix (12.5 μl), real-time qPCR was performed. Quantitative real-time PCR reaction was performed with the 7000 Sequence Detection System (ABI). Relative expressions were calculated using the formula $2^{-\Delta\Delta CT}$ values ($\Delta Ct=Ct^{gene}-Ct^{control}$). The primer sequences and PCR conditions are summarized in Table I.

miRNA target gene prediction. The target gene prediction software TargetScan was used to analyze differentially expressed miRNA (TargetScan human V6).

miRNA-mRNA correlation analysis. Negative regulation underlines the miRNA-mRNA relationship. We performed negative correlation analysis of significant expression patterns. We focused on the intersection of the 6444 miRNA target genes and the 2049 mRNA gene.

Gene ontology analysis of inversely related target genes. We first mapped target genes to each node of the gene ontology (GO) database. The analysis was carried out using the software DAVID (http://david.abcc.ncifcrf.gov/), according to the statistical test method (P-value) of significantly enriched categories ($P\leq0.01$ as a result of the final output).

Pathway analysis of inversely related target genes. We first mapped target genes to the KEGG pathway database. We used the software DAVID for analysis (http://david.abcc.ncifcrf.gov/). $P\leq0.05$ was regarded as statistically significant.

Network analysis of inversely related target genes

Functional regulatory network of miRNA target genes: the miRNA-GO-network. The target gene cluster was classified in

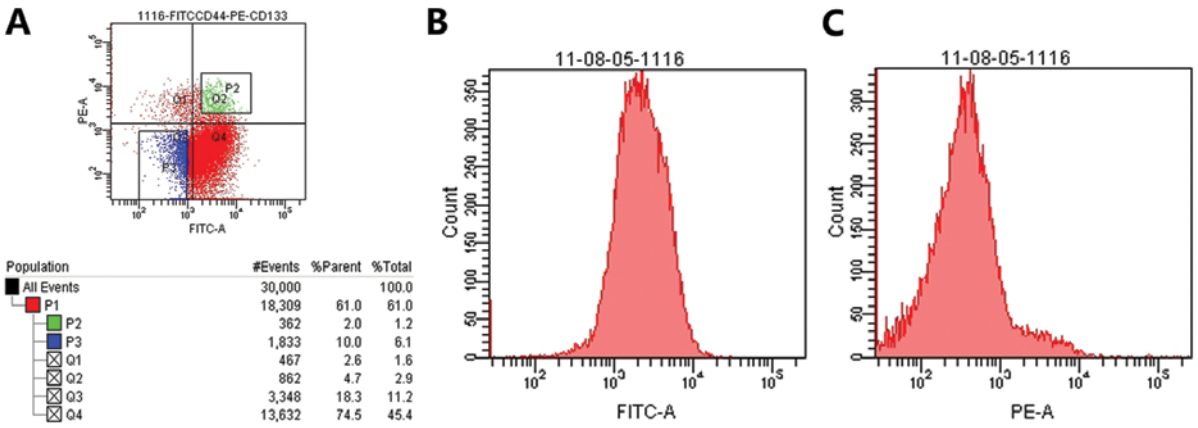


Figure 1. Isolation and characterization of colon stem cells in the SW1116 cell line. (A) CD44 was analyzed using FITC-labeled antibody. CD133 was analyzed using PE-labeled antibody. Top, methodology of flow cytometry cell sorting in the SW1116 cell line. P2, $CD44^{+}CD133^{+}$ sorting area; P3, $CD44^{-}CD133^{-}$ sorting area. P2 accounted for only 1.2% of the total number of cells; P3 accounted for only 6.1% of the total number of cells. (B and C) The expression of cell surface CD44 and CD133 antibody following amplification.

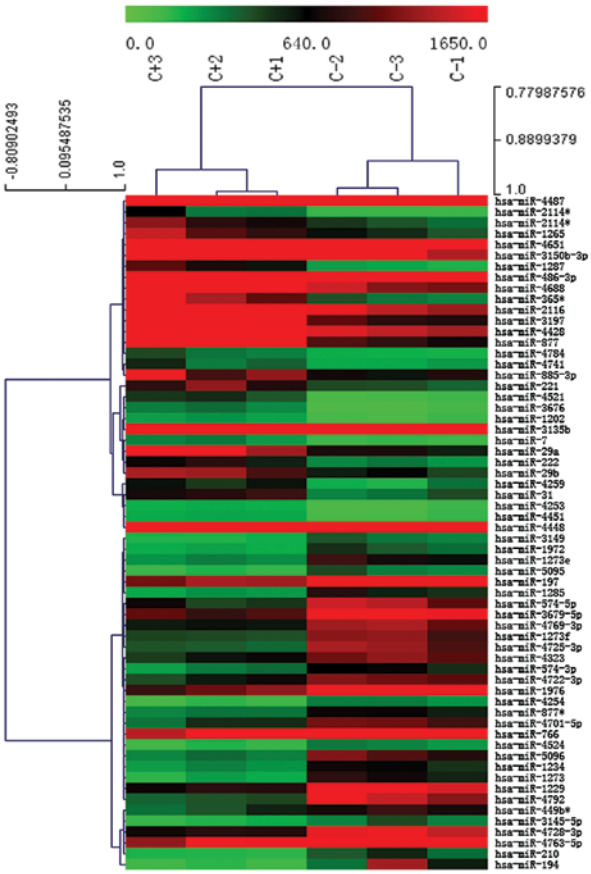
Table I. Sequences of primers and parameters for reverse-transcription and real-time PCR.

	Forward primer (5'-3')	Reverse primer (5'-3')
MiR-29a	ACACTCCAGCTGGGTAGCACCATCTGAAAT	Kit provides
MiR-29b	ACACTCCAGCTGGGTAGCACCATTTGAAATC	Kit provides
MiR-449b	ACACTCCAGCTGGGAGGCAGTGTATTGTTA	Kit provides
MiR-4524a	ACACTCCAGCTGGGATAGCAGCATGAACCT	Kit provides
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
NRAS	TGAAACCTCAGCCAAGACCAGACA	TGGCAATCCCATACAACCCTGAGT
FOS	TGTCTGTGGCTTCCCTTGATCTGA	TGGATGATGCTGGGAACAGGAAGT
WASF2	AGATGCTGCAGGACACCAAGGATA	ACCAAAGTGGGTGGATACCCAGAA
COL5A1	TGCTCCAGGGATTCCTTCAAGGTT	ATAGGAGAGCAGTTTCCCACGCTT
CDK6	TGCACAGTGTCACGAACAGACAGA	TTAGATCGCGATGCACTACTCGGT
CCND1	AGAAGCTGTGCATCTACACCGACA	TGATCTGTTTGTTCTCCTCCGCCT
E2F3	AGTTCATTCAGCTCCTGAGCCAGT	CAGCCCATCCATTGGACGTTGTTT
GNG12	AGCACCAACAATATAGCCCAGGCA	ACTCCTGGCATGTTCCTCACAGTA
GNA12	TCAAGAAGCACTTCCCGGACTTCA	TTTCACAGCATGGAACACGAAGCG
β-actin	ACCAACTGGGACGACATGGAGAAA	TAGCACAGCCTGGATAGCAACGTA

accordance with the classification of the GO BP. miRNA-GO network was built. The network reflects the target miRNA target gene function. Network eigenvalue (degree) was calculated according to the location of each miRNA function. The miRNAs with the highest eigenvalues which regulate a number of gene functions and the sample status, are always located in pivotal positions in the network.

miRNA-gene network. Using the Sanger miRNA database, we screened target genes regulated by differentially expressed miRNAs (TargetScan). Then we took the differentially expressed genes and the intersection of target genes and differentially expressed genes, which is differential target genes regulated by differential miRNA. Classifying this part of differential target genes regulated by differential miRNA with GO BP, we found that significantly differential target genes belong to significant GO ($P<0.01$). Using the relationship between miRNA and target gene, we built the miRNA-gene network.

Network of significant, differentially expressed target genes: miRNA-path network. With pathway analysis results, we built the pathway network. The network reflects the relationship between miRNAs and pathways. We calculated the specific degree according to the location of each miRNA and pathways in the network. miRNAs and pathways with the highest degree are always located in pivotal positions in the network.



Upregulated	Ration	Log2	Downregulated	Ration	Log2
hsa-miR-4487	3.94	1.98	hsa-miR-3149	0.45	-1.15
hsa-miR-2114*	3.46	1.79	hsa-miR-1972	0.47	-1.08
hsa-miR-2114*	2.14	1.10	hsa-miR-1273e	0.37	-1.44
hsa-miR-1265	2.10	1.07	hsa-miR-5095	0.40	-1.32
hsa-miR-4651	2.03	1.02	hsa-miR-197	0.39	-1.35
hsa-miR-3150b-3p	3.92	1.97	hsa-miR-1285	0.36	-1.49
hsa-miR-1287	3.77	1.92	hsa-miR-574-5p	0.41	-1.28
hsa-miR-486-3p	4.18	2.06	hsa-miR-3679-5p	0.49	-1.03
hsa-miR-4688	3.33	1.74	hsa-miR-4769-3p	0.46	-1.12
hsa-miR-365*	3.86	1.95	hsa-miR-1273f	0.38	-1.38
hsa-miR-2116	2.33	1.22	hsa-miR-4725-3p	0.33	-1.61
hsa-miR-3197	2.90	1.54	hsa-miR-4323	0.47	-1.09
hsa-miR-4428	2.63	1.40	hsa-miR-574-3p	0.43	-1.20
hsa-miR-877	3.97	1.99	hsa-miR-4722-3p	0.46	-1.12
hsa-miR-4784	2.02	1.02	hsa-miR-1976	0.43	-1.22
hsa-miR-4741	2.07	1.05	hsa-miR-4254	0.41	-1.27
hsa-miR-885-3p	2.02	1.01	hsa-miR-877*	0.48	-1.05
hsa-miR-221	2.27	1.18	hsa-miR-4701-5p	0.40	-1.32
hsa-miR-4521	4.77	2.25	hsa-miR-766	0.35	-1.51
hsa-miR-3676	4.00	2.00	hsa-miR-4524	0.47	-1.09
hsa-miR-1202	2.55	1.35	hsa-miR-5096	0.32	-1.66
hsa-miR-3135b	2.46	1.30	hsa-miR-1234	0.43	-1.22
hsa-miR-7	2.33	1.22	hsa-miR-1273	0.27	-1.90
hsa-miR-29a	2.35	1.24	hsa-miR-1229	0.39	-1.35
hsa-miR-222	2.32	1.22	hsa-miR-4792	0.27	-1.91
hsa-miR-29b	2.14	1.10	hsa-miR-449b*	0.49	-1.04
hsa-miR-4259	2.94	1.56	hsa-miR-3145-5p	0.42	-1.24
hsa-miR-31	2.29	1.20	hsa-miR-4728-3p	0.42	-1.25
hsa-miR-4253	2.17	1.12	hsa-miR-4763-5p	0.41	-1.30
hsa-miR-4451	2.05	1.03	hsa-miR-210	0.36	-1.49
hsa-miR-4448	2.01	1.01	hsa-miR-194	0.17	-2.53

Figure 2. Differential miRNA profile expression in colon cancer stem and non-stem cells. All 31 upregulated and 31 downregulated miRNAs are listed. The degree of differentially expressed miRNAs compared to non-stem cells is listed in the table.

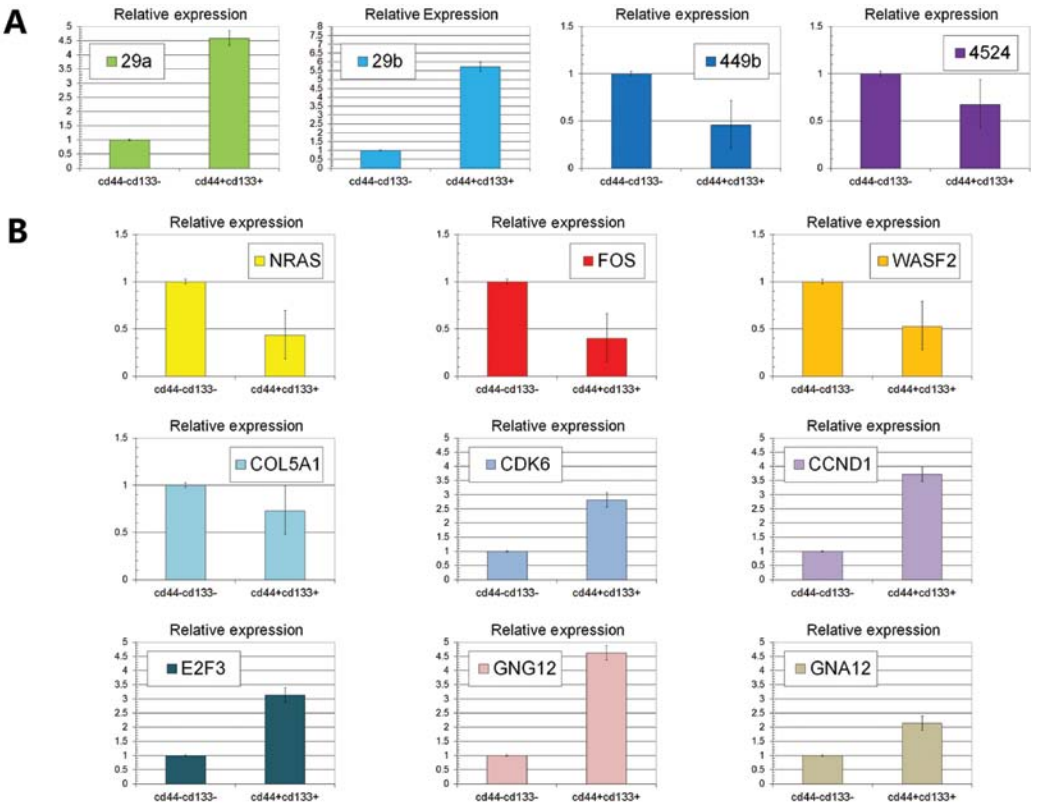


Figure 3. (A) The expression levels of miR29a, miR29b, miR449b and miR4524 were detected using RT-PCR. We found that miR29a and miR29b were upregulated while miR449b and miR4524 were downregulated in colon stem cells compared with non-stem cells (P<0.05). (B) The expression levels of NRAS, FOS, WASF2, COL5A1, CDK6, CCND1, E2F3, GNG12 and GNA12 were detected using RT-PCR. NRAS, FOS, WASF2 and COL5A1 were downregulated in colon stem cells (P<0.05). On the contrary, CDK6, CCND1, E2F3, GNG12 and GNA12 were upregulated (P<0.05).

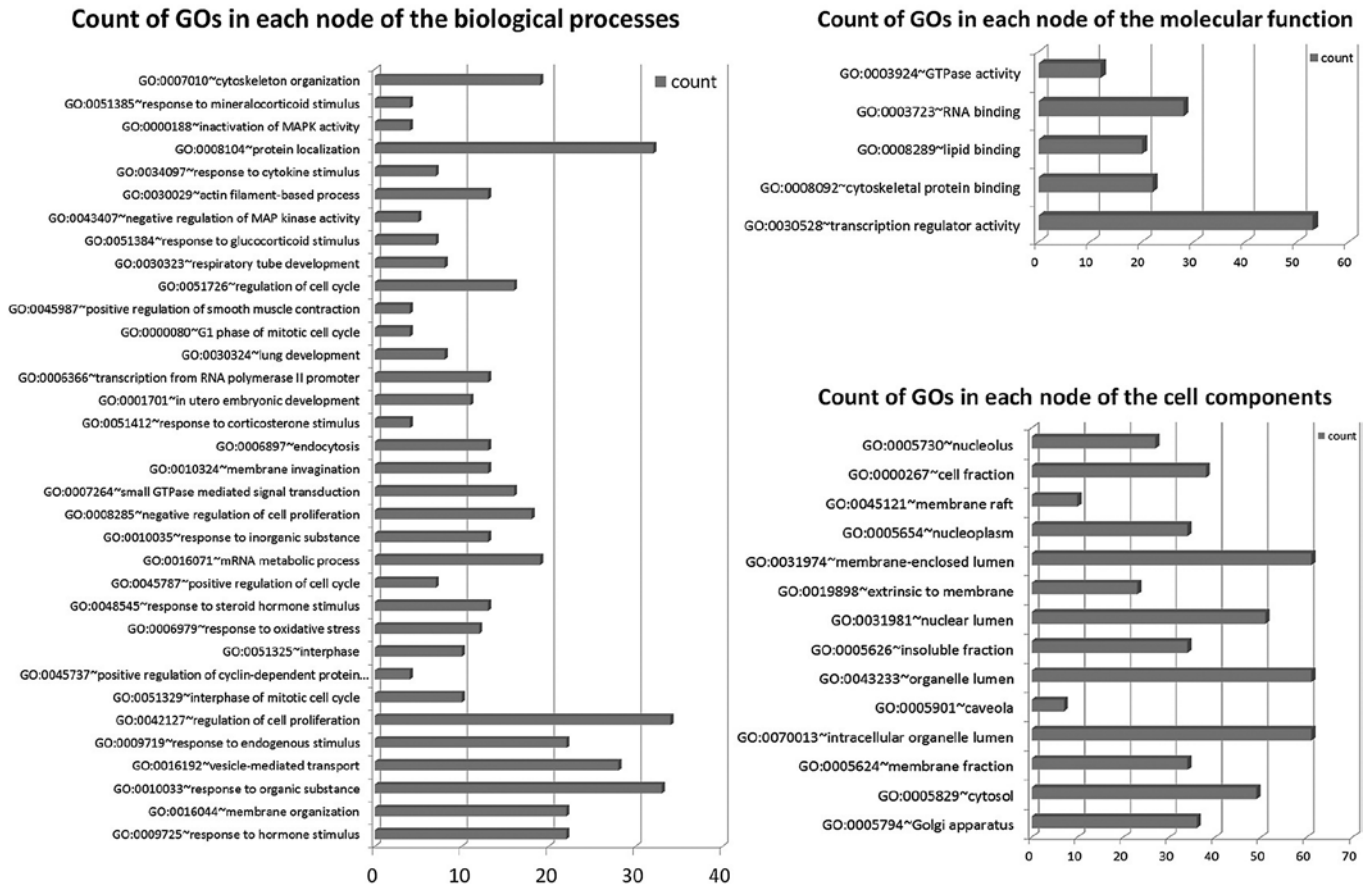


Figure 4. Differentially expressed mRNAs regulated by differentially expressed miRNAs involving a total of 34 significant GOs in the biological process, 5 GOs in molecular function and 14 GOs in cellular component. Counts indicate enriched levels of genes acting on notes by the statistical test method (P≤0.01).

Results

Differential miRNA expression profile in colon CSCs and non-stem cells. We used an array-based miRNA chip to investigate the differential miRNA expression profile in colon CSCs and non-stem cells. A total of 1711 human miRNAs were examined. There are 31 miRNAs significantly upregulated and 31 miRNAs significantly downregulated. Of these, miR-4521 was the most significantly upregulated miRNA. Similarly, miR-194 was the most significantly downregulated miRNA (Fig. 2).

Differential mRNA expression profile in colon CSCs and non-stem cells. We used mRNA chip to investigate the differential mRNA expression profile in colon CSCs and non-stem cells. We examined 34694 mRNAs. There are a total of 2049 differentially expressed mRNAs detected (Fig. 3).

GOs are regulated by miRNAs. We found that GOs were significantly regulated by differentially expressed genes in the biological process, molecular function and cellular component. Results show differentially expressed mRNAs regulated by differentially expressed miRNAs involving a total of 34 significant GOs in the biological process, 5 GOs in the molecular function and 14 GOs in the cellular component. The most differentially expressed GOs in the biological process are hormone stimulus, membrane organization, response to organic substance, vesicle-mediated transport, endogenous stimulus, regulation of cell proliferation, interphase of mitotic cell cycle, positive regulation of cyclin-dependent protein kinase activity and regulation of cell cycle. The most differentially expressed GOs in molecular function are transcription regulator activity, cytoskeletal protein binding, lipid binding, RNA binding and GTPase activity. The most differentially expressed GOs in cellular component are golgi apparatus, cytosol, membrane fraction and intracellular organelle lumen. Genes were classified in accordance with the GO biological process, and we constructed the miRNA-GO-network. miRNAs in different locations of the network have a different degree. The miRNA which has the highest degree is located in the central position of the network. From the network, we find that hsa-miR-29a, hsa-miR-29b, hsa-miR-449b, hsa-miR-4524 and hsa-miR-7 are involved in more GOs related to the characteristics of the sample. Regulation of cell proliferation, vesicle-mediated transport, response to organic substance and protein localization are important GOs represented by miRNA target genes (Figs. 4 and 5).

Signaling pathways are regulated by miRNAs. We used the KEGG database to analyze target genes. The target genes regulated by differentially expressed miRNAs involving a

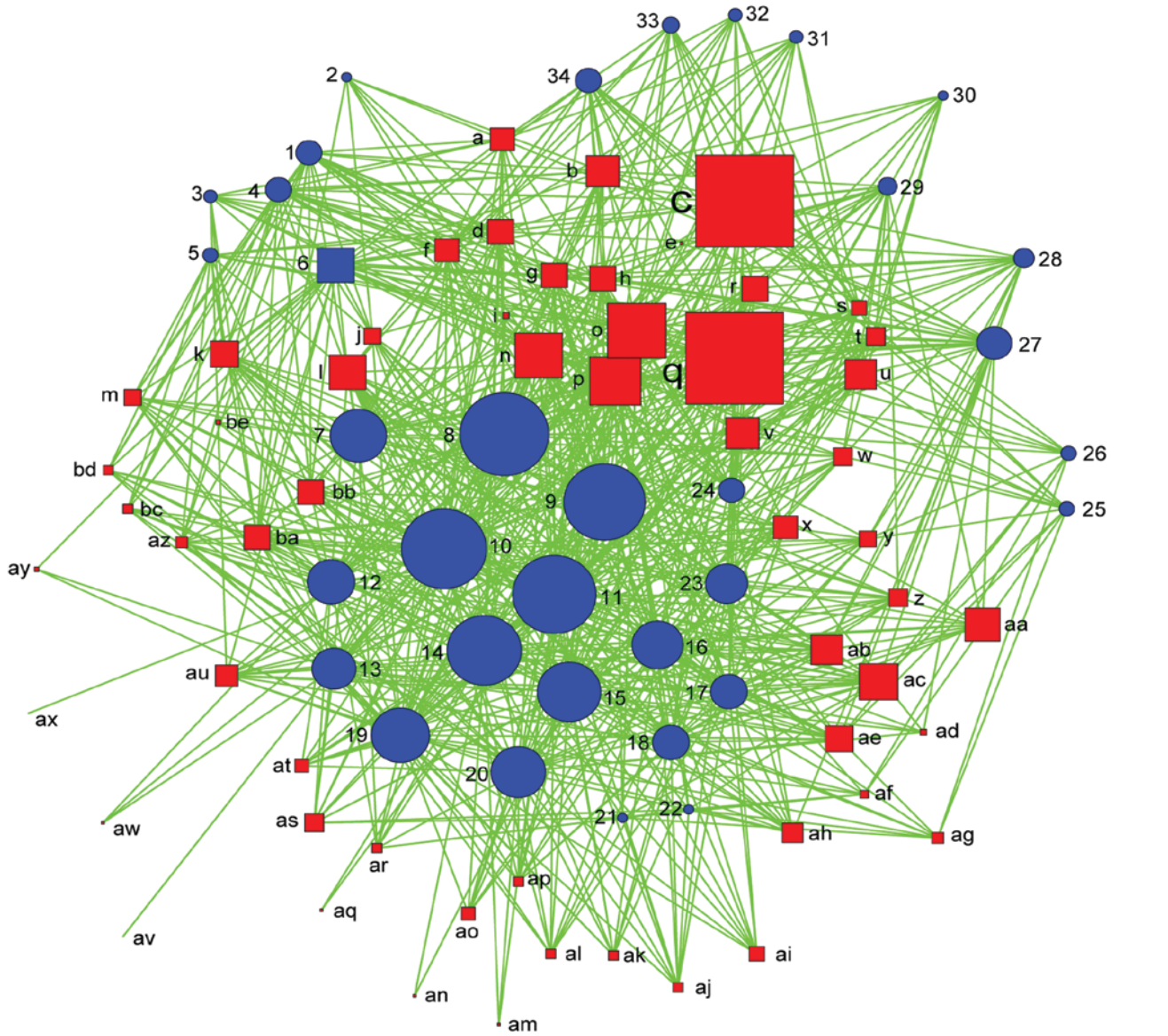


Figure 5. A network diagram showing the relationship between miRNAs and GOs. The rectangles represent miRNAs, the circles represent the GO functional annotation, a straight line indicates the regulation of relations between the miRNA and GO. The more GO miRNA was regulated, the greater its area; similarly, the more GO was regulated by miRNA, the greater its area.

total of 18 significant pathways, containing 47 genes. These pathways include pathways in cancer, endocytosis, regulation of actin cytoskeleton, the VEGF signaling pathway, the insulin signaling pathway, colorectal cancer and RNA degradation. Seen from the significant pathway relationship network, the main signal pathways are CRC, chronic myeloid leukemia, glioma, regulation of actin cytoskeleton, endocytosis and the VEGF signaling pathway, of which regulation of actin cytoskeleton and pathways in cancer have the largest degree. miRNA-29a, miRNA-449b, miRNA-29b, miRNA-4524, miRNA-194 are at the key position in the miRNA-pathway-network. This is essentially the same as the result of the miRNA-GO-network (Figs. 6 and 7).

Regulation network of differentially expressed miRNAs. There are 62 miRNAs, 34 GOs, 18 pathways that have significant

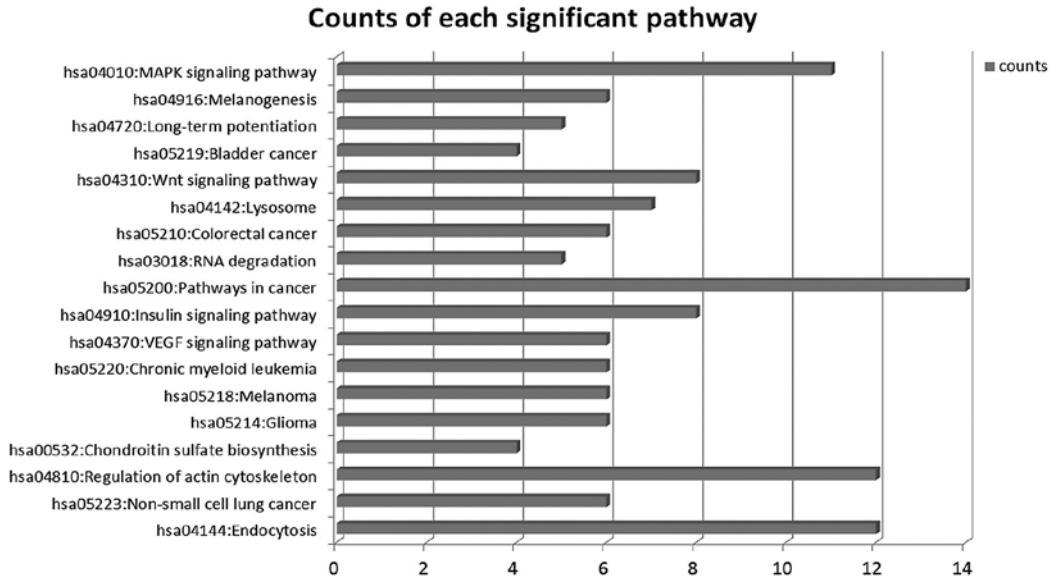


Figure 6. The target genes regulated by differentially expressed miRNAs involving a total of 18 significant pathways, containing 47 genes. Counts indicate enriched levels of pathways acting in keeping characteristics of colon stem cells by the statistical test method (P≤0.1).

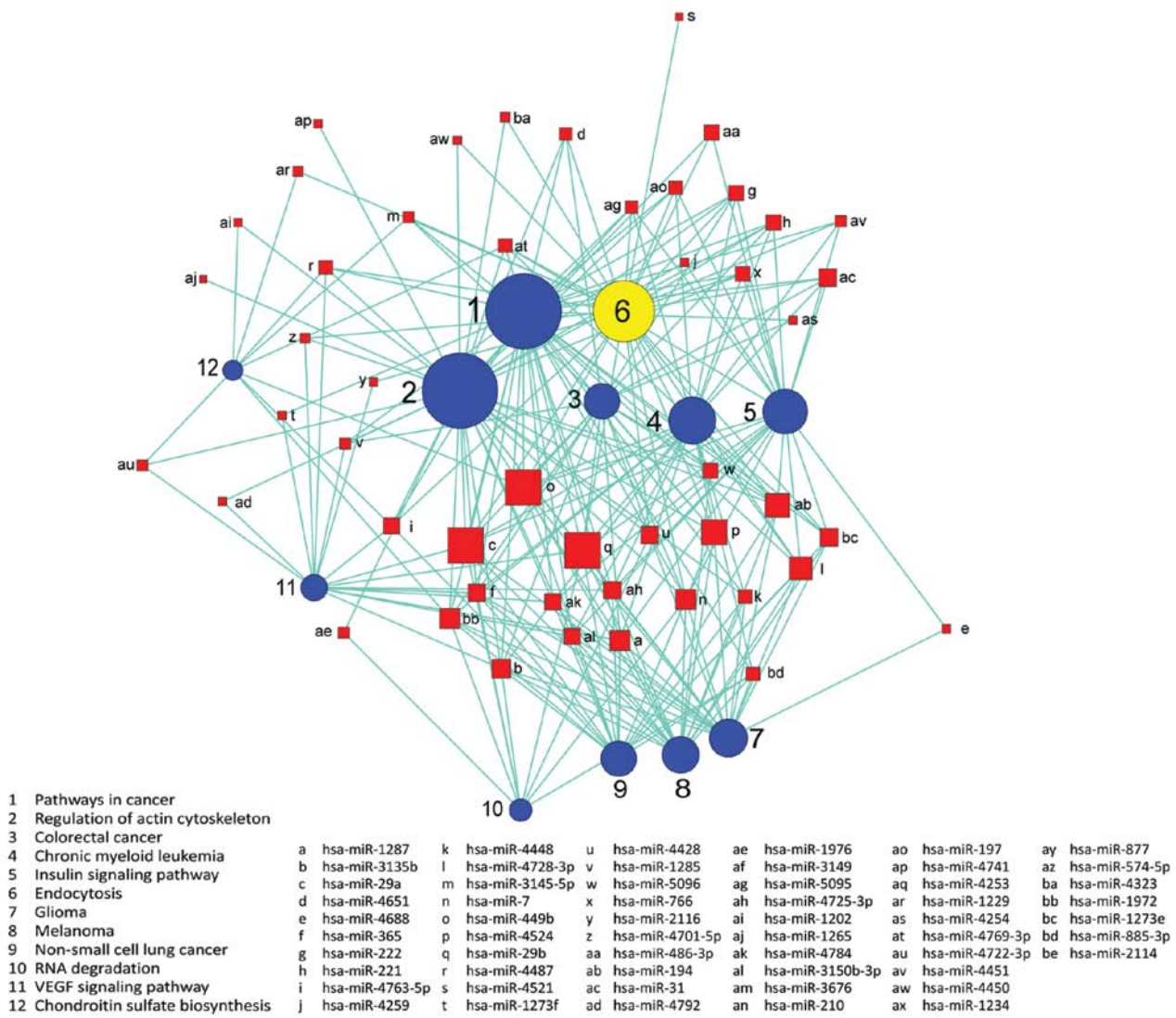
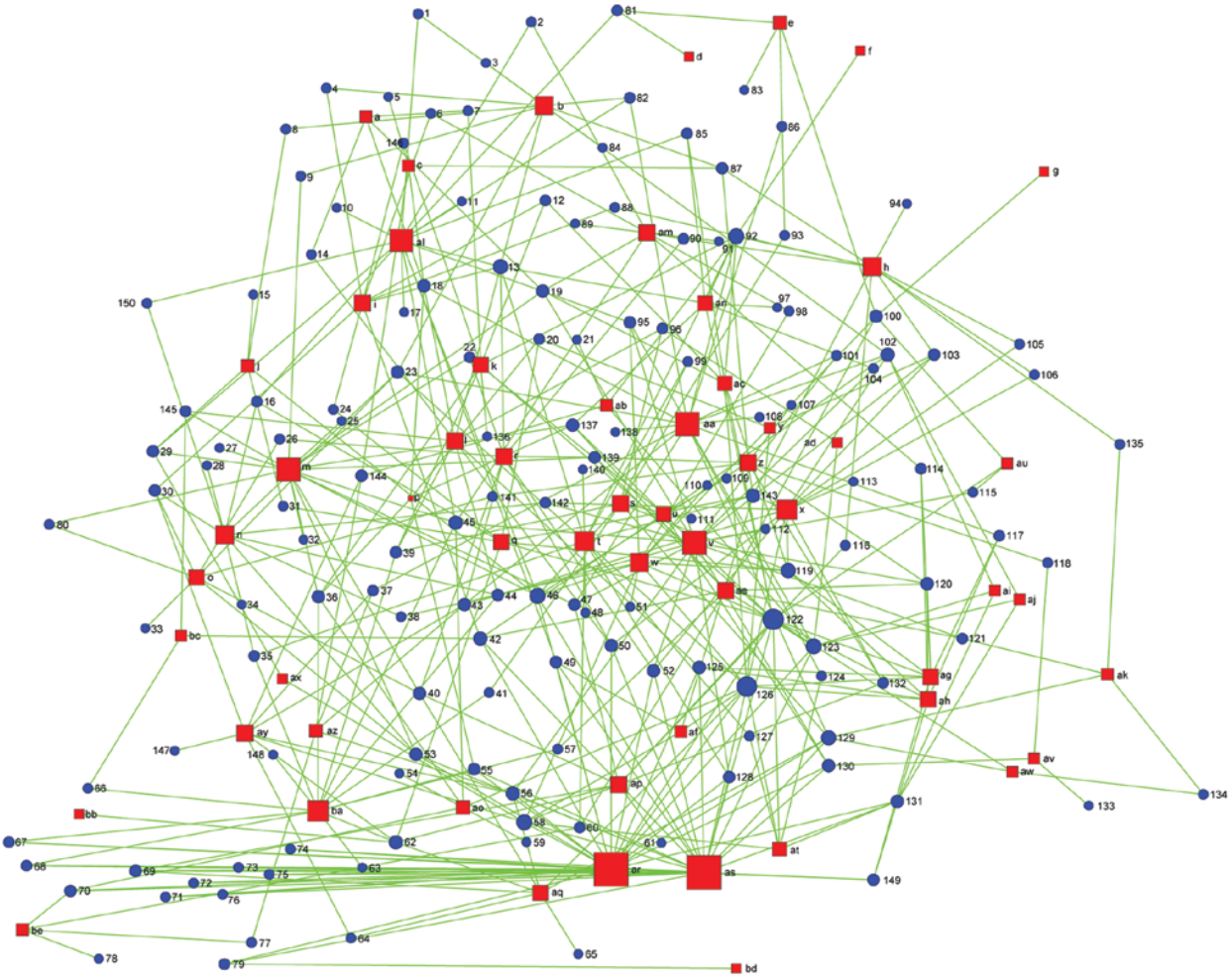


Figure 7. A network diagram showing the relationship between miRNAs and pathways. The rectangles represent miRNAs, the circles represent the pathways, the straight lines represent the regulation of relations between the miRNAs and the pathways. The more the gene miRNA was regulated, the greater its area; similarly the more the pathways was regulated by miRNAs, the greater its area.



1	GMEB2	16	CLASP1	31	CTGF	46	RAB6B	61	NUP62	76	ADA	91	FOXF1	106	RND3	121	RAPGEF2	136	MFGE8
2	SLC30A1	17	RAB13	32	CEBPA	47	SPRED1	62	ATG7	77	PPP1CB	92	SAR1A	107	TUBGCP5	122	SYNCRIP	137	RAB5B
3	PCGF2	18	VPS37D	33	EZR	48	TRPC4AP	63	OXR1	78	APLP1	93	PPP2CA	108	PPP1R10	123	AFF4	138	LMAN2L
4	YBX2	19	MYO5A	34	PPP3CB	49	SIX5	64	ENO2	79	NAPB	94	HSPA4	109	WIBG	124	SERPINE1	139	SF3B3
5	CHMP4B	20	DUSP16	35	SNCA	50	DCP2	65	VCP	80	SHROOM3	95	E2F3	110	SLC25A36	125	FOS	140	RAB31
6	PALLD	21	XPO6	36	EIF4EBP2	51	TCF3	66	SERP1	81	NACC2	96	CALM1	111	CAV1	126	WASF2	141	KHSRP
7	FNBP1	22	RBM12	37	PACS1	52	XPO4	67	PAN2	82	SYVN1	97	EIF2C2	112	ARL2	127	GDI1	142	GNA12
8	PTGS2	23	VAMP2	38	POLR2F	53	COL5A1	68	CNOT8	83	PAPOLA	98	CXADR	113	RAB10	128	VPS37C	143	SLC9A1
9	VAMP4	24	DNAJC8	39	IRS2	54	SPHK2	69	LASP1	84	HMGB2	99	SH3KBP1	114	BCL2L11	129	CDK6	144	PXN
10	CRK	25	LRP10	40	ADCY6	55	KLF4	70	MCL1	85	PAFAH1B1	100	TCEB3	115	PRDM4	130	AP4E1	145	RABL3
11	NUDC	26	OBFC2A	41	CNN3	56	GNG12	71	TPM1	86	NOTCH1	101	FOSL1	116	RBM3	131	NRAS	146	NUPR1
12	POM121C	27	TESK2	42	FOXO4	57	PIM1	72	CPS1	87	B4GALT7	102	GRSF1	117	CDKN1C	132	SKAP2	147	TPD52L1
13	SSH2	28	M6PR	43	CCND1	58	NFIX	73	CAV2	88	HCFC1	103	HNRNPA1	118	NQO1	133	SIK1	148	EGFR
14	PTGS1	29	RBBP4	44	BMPR2	59	NSF	74	BTG2	89	NEK2	104	HNRNPD	119	HNRNPA3	134	RAB35	149	ROD1
15	PRPF19	30	SRF	45	TFRC	60	CYTH2	75	LOX	90	CHP	105	AP1S1	120	GNAI3	135	RNMT	150	NRP1

a	hsa-miR-4521	p	hsa-miR-1229	ae	hsa-miR-4323	at	hsa-miR-4725-3p
b	hsa-miR-4487	q	hsa-miR-5095	af	hsa-miR-4741	au	hsa-miR-877
c	hsa-miR-4763-5p	r	hsa-miR-365	ag	hsa-miR-221	av	hsa-miR-2114
d	hsa-miR-1234	s	hsa-miR-4728-3p	ah	hsa-miR-222	aw	hsa-miR-1273e
e	hsa-miR-3149	t	hsa-miR-486-3p	ai	hsa-miR-4784	ax	hsa-miR-4450
f	hsa-miR-3676	u	hsa-miR-3145-5p	aj	hsa-miR-3150b-3p	ay	hsa-miR-3135b
g	hsa-miR-4451	v	hsa-miR-4524	ak	hsa-miR-885-3p	az	hsa-miR-1287
h	hsa-miR-4722-3p	w	hsa-miR-4448	al	hsa-miR-194	ba	hsa-miR-7
i	hsa-miR-4651	x	hsa-miR-1976	am	hsa-miR-2116	bb	hsa-miR-210
j	hsa-miR-1273f	y	hsa-miR-1265	an	hsa-miR-197	bc	hsa-miR-574-5p
k	hsa-miR-766	z	hsa-miR-4769-3p	ao	hsa-miR-4701-5p	bd	hsa-miR-4253
l	hsa-miR-4428	aa	hsa-miR-449b	ap	hsa-miR-4254	be	hsa-miR-4259
m	hsa-miR-31	ab	hsa-miR-4792	aq	hsa-miR-1972		
n	hsa-miR-1285	ac	hsa-miR-1202	ar	hsa-miR-29a		
o	hsa-miR-5096	ad	hsa-miR-4688	as	hsa-miR-29b		

Figure 8. A network diagram showing the relationship between miRNAs and genes. The rectangles represent miRNAs, the circles represent genes, the straight lines represent the regulation of relations between miRNAs and genes. The more the gene miRNA was regulated, the greater its area; the more the gene was regulated by miRNAs, the greater its area.

differences between colon CSCs and non-stem cells. Therefore, regulatory networks of differentially expressed miRNAs displaying the miRNAs with the highest degree affected the surrounding genes hsa-mi-29a, hsa-miR-29b, hsa-miR-4524, hsa-miR-449b, and hsa-miR-31 confirming previous results. Details are shown in Fig. 8.

Discussion

Cancer microRNA (miRNA) expression profiling has been widely reported. Tumors of various organs in the body have corresponding miRNA expression profile changes. Therefore, miRNAs, as regulators of gene expression, are involved in the tumor development process, acting as oncogenes or tumor suppressor genes (17-20). Changes in miRNA expression profile are found at various stages of colon tumor development. Li *et al* reported that overexpression of miR-203 can significantly decrease cell proliferation and survival, and induce cell apoptosis in the p53-mutated colorectal cancer (CRC) cells (21). Strillacci *et al* demonstrated that downregulation of the miR-101 level could represent one of the leading causes of COX-2 overexpression in CRC cells (22). Li *et al* also reported that miR-181b can suppress proliferation of U87 glioma stem cells. Overexpression of miR-181b can reduce chemoresistance to temozolomide in U87 glioma stem cells (23). Therefore, these data show that miRNAs are related to the characteristics of tumor cells. Li *et al* recently found expression of breast cancer resistance protein BCRP/ABCG2 regulatory miRNAs (hsa-miR-328, -519c and -520h) in stem-like ABCG2$^+$ cancer cells (24). Zhang *et al* identified a colon cancer stem cell (CSC) miRNA signature comprising a total of 19 differentially expressed miRNAs, such as miR-429, miR-155, and miR-320d, in the HT29 adenocarcinoma cell line (25). The above show that the special phenotype and biological characteristics of CSCs are results of miRNA regulation. However, differences in expression profiles of miRNA between colon CSCs and non-stem cells and the relationship between differential miRNA expression and the function of stem cells are rarely reported. Therefore, in our study, expression profiling of 1711 miRNAs based on OneArray microarray platform and 34694 mRNAs based on Illumina Whole-Genome system of samples of stem cells and non-stem cells of the SW1116 human CRC cell line was carried out to further explore the characteristics of CRC. The array data were confirmed using RT-PCR. We also classified colon stem cells with the profiling of miRNA. By defining miRNAs that are related to the characteristics of colon CSCs we will be able to further clarify the regulatory pathway and gain insight into the features of colon CSCs.

The results, by microarray analysis in the SW1116 CRC cell line, showed 62 miRNAs and 2049 mRNAs differentially expressed in colon stem cells compared to non-stem cells. Among these differentially expressed miRNAs, 31 miRNAs represented overexpression in colon stem cells, whereas the remaining 31 miRNAs demonstrated underexpression. Among these miRNAs, overexpression of mir-29a, mir-29b and underexpression of mir-449b, mir4524 were confirmed by quantitative RT-PCR assay, proving the chip results. This miRNA expression profiling indicates characteristics of colon CSCs. Thus, miRNA regulation is intricately related to distinctive features and the biological performance of colon CSCs.

Gene cluster controlling the cell cycle gene cluster is extremely important for the maintenance of stem cell growth and proliferation characteristics. GO analysis showed that there is a noticeable change (PPP1CB, CCND1, CDKN1C and CDK6) in cell cycle-related (GO: 0051329, interphase of mitotic cell cycle; GO: 0045787, positive regulation of cell cycle; GO: 0000080, G1 phase of mitotic cell cycle; GO: 0051726, regulation of cell cycle) gene cluster. This shows that the change of cell cycle of stem cells is key to maintaining its important characteristics, and this distinguishes stem cells from non-stem cells. This feature gives stem cells their characteristics and their ability to proliferate and metastasize.

The change in the characterization of cell differentiation is an important feature of tumor stem cells that differentiates them from non-stem cells. Numerous studies have shown that the change of cell differentiation plays a crucial role in tumor occurrence, development and metastasis. We found that GO 0042127 (regulation of cell proliferation) related genes have significantly altered. Thirty-four genes correspond to this GO, including CAV1, KLF4, SERPINE1 and BMPR2. This reflects the need of the stem cells to maintain the level of the high-density proliferation, in order to progress to distant metastases, cell proliferation-related gene expression was significantly changed.

Many signal transduction pathways in stem cells also changed. The most important change is the MAPK signaling pathway. MAPK, downstream signaling molecules with serine and threonine protein kinase activity in the Ras pathway, can format AP-1 acting on the nucleus to activate specific genes in order to pass the signal by activating the C-Fos, C-Jun transcription regulator. The MAPK signal transduction pathway plays an important role in stress responses such as inflammation and apoptosis. MAPK can promote endothelial cell proliferation and angiogenesis. Tumor angiogenesis can provide more nutrients to accelerate the growth of the tumor and to promote the proliferation of cancer cells. The MAPK pathway plays a unique role in the growth of the tumor stem cell and its transfer characteristics (26). The Wnt pathway is also involved. The majority of downstream target genes of the Wnt pathway is involved in cell proliferation and apoptosis genes. Playing a key role in the embryo during development, the Wnt pathway occurs with a variety of human tumors, especially in CRC. In colon CSCs, Wnt pathway-related genes (FOSL1, CCND1, CHP, FZD9 FZD4, PPP3CB PPP2CA, TBL1X) change (27). In addition, the Jak-STAT signaling pathway, ErbB signaling pathway, VEGF signaling pathway also showed varying degrees of change in colon CSCs.

Changes in cytoskeletal proteins may also be involved in stem cells. We found that the pathway of regulation of actin cytoskeleton is involved in a significant change. Kasper *et al* found that actin cytoskeleton is involved in mesenchymal stem cell aging (28). Cell-matrix adhesion is closely related to a variety of cellular processes such as cell migration, cell differentiation, and cell proliferation. Pathways such as focal adhesion, gap junction, adherens junction and tight junction are also involved.

In conclusion, by analyzing the difference of miRNA and mRNA expression in colon CSCs and non-stem cells, we found that miRNAs play an important role in the expression of stem cell characteristics. This study provides a new perspective on CRC metastasis and recurrence and the findings of this study may contribute to the treatment and diagnosis of CRC.

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References

1. Jemal A, Murray T, Ward E, *et al*: Cancer statistics. CA Cancer J Clin 59: 225-249, 2009.
2. Lengauer C, Kinzler K and Vogelstein B: Genetic instabilities in human cancers. Nature 396: 643-649, 1998.
3. Baylin S and Ohm J: Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 6: 107-116, 2006.
4. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 8: 286-298, 2007.
5. Huang E and Wicha M: Colon cancer stem cells: implications for prevention and therapy. Trends Mol Med 14: 503-509, 2008.
6. Pardal R, Clarke M and Morrison S: Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3: 895-902, 2003.
7. Tan B, Park C, Ailles L and Weissman I: The cancer stem cell hypothesis: a work in progress. Lab Invest 86: 1203-1207, 2006.
8. Chen Y, Hsu H, Chen Y, *et al*: Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. PLoS One 3: e2637, 2008.
9. Gangemi R, Griffero F, Marubbi D, *et al*: SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells 27: 40-48, 2009.
10. Todaro M, Alea M, Stefano DA, *et al*: Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 1: 389-402, 2007.
11. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism and function. Cell 116: 281-297, 2004.
12. Gregory RI, Yan KP, Amuthan G, *et al*: The microprocessor complex mediates the genesis of microRNAs. Nature 432: 235-240, 2004.
13. Bernstein E, Caudy A, Hammond SM and Hannon GJ: Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363-366, 2001.
14. Ambros V: The functions of animal microRNAs. Nature 431: 350-355, 2004.
15. Meister G and Tuschl T: Mechanisms of gene silencing by double-stranded RNA. Nature 431: 343-349, 2004.
16. Peters L and Meister G: Argonaute proteins: mediators of RNA silencing. Mol Cell 26: 611-623, 2007.
17. Harfe BD, McManus M, Mansfield JH, Hornstein E and Tabin CJ: The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci USA 102: 10898-10903, 2005.
18. Murchison EP, Stein P, Xuan ZY, *et al*: Critical roles for Dicer in the female germline. Genes Dev 21: 682-693, 2007.
19. Wang Y, Medvid R, Melton C, Jaenisch R and Blelloch R: DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. Nat Genet 39: 380-385, 2007.
20. Wienholds E, Koudijs MJ, Eeden F, Cuppen E and Plasterk R: The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35: 217-218, 2003.
21. Li J, Chen YX, Zhao JF, Kong FR and Zhang YD: miR-203 reverses chemoresistance in p53-mutated colon cancer cells through downregulation of Akt2 expression. Cancer Lett 304: 52-59, 2011.
22. Strillacci A, Griffonia C, Sansone P, *et al*: MiR-101 downregulation is involved in cyclooxygenase-2 overexpression in human colon cancer cells. Exp Cell Res 315: 1439-1447, 2009.
23. Li P, Lu XM, Wang YY, *et al*: MiR181-b suppresses proliferation of and reduces chemoresistance to temozolomide in U87 glioma stem cells. J Biomed Res 24: 436-443, 2010.
24. Li X, Pan YZ, Seigel GM, *et al*: Breast cancer resistance protein BCRP/ABCG2 regulatory microRNAs (hsa-miR-328, -519c and -520h) and their differential expression in stem-like ABCG2$^+$ cancer cells. Biochem Pharmacol 81: 783-792, 2011.
25. Zhang HL, Li WH, Nan FF, *et al*: MicroRNA expression profile of colon cancer stem-like cells in HT29 adenocarcinoma cell line. Biochem Biophys Res Commun 404: 273-278, 2011.
26. Seger R and Krebs EG: The MAPK signaling cascade. FASEB J: 726-735, 1995.
27. Polakis P: Wnt signaling and cancer. Genes Dev 14: 1837-1851, 2000.
28. Kasper G, Mao L, Geissler S, Draycheva A, Trippens J, Kühnisch J, Tschirschmann M, Kaspar K, Perka C, Duda GN and Klose J: Insights into mesenchymal stem cell aging: involvement of antioxidant defense and actin cytoskeleton. Stem Cells 27: 1288-1297, 2009.