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Original Article

Identifying specific miRNAs and associated mRNAs in CD44 and CD90 cancer stem cell subtypes in gastric cancer cell line SNU-5

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Abstract: Cancer stem cells (CSCs) are capable of generating multiple types of cells and play a vital role in promoting gastric cancer (GC) progression. Our previous research indicated that gastric CSCs with surface markers of CD44+ were more invasive compared to CD44- CD90+ CSCs (CD90+ CSCs), whereas CD90+ CSCs exhibited higher levels of proliferation than CD44+ CSCs. However, the mechanism and characteristics of marker-positive gastric CSCs are poorly understood. In this study, we profiled expression of miRNAs and mRNAs in CD44+ CSCs, CD90+ CSCs, and CD44- CD90- cell subtype (control) from SNU-5 cells by microarray analysis. Our results suggested some specially expressed miRNA-mRNA pairs in CD44+ and CD90+ CSCs. We performed Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to analyze the correlation and function of those pairs. We also validated the pairs that may play roles in metastasis by qRT-PCR. In CD44+ CSCs, we observed hsa-miR-15b-5p was up-regulated and its target genes AMOT, USP31, KALRN, EPB41L4B, ATP2B2, and EMC4 were down-regulated, which may relate to invasion and migration. In CD90+ CSCs, we observed hsa-miR-3631-3p is up-regulated, while its target genes QKI, TRIM67 and HMGA2 are down-regulated, which is associated with proliferation. We also found that hsa-miR-1910-5p is up-regulated while its target gene QKI and HMGA2 are down-regulated in CD90+ CSCs. The screened miRNA-mRNA pairs give us new insight into the mechanism of different phenotypes and biomarkers capable of identifying and isolating metastatic and tumorigenic CSCs. Those miRNA-mRNA pairs may also act as treatment for GC.

Keywords: Gastric cancer (GC), cancer stem cells (CSCs), miRNAs, CD44, CD90

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer related mortality, particularly in East Asia, Eastern Europe, and South America [1]. Several factors (environmental and genetic) were reported to play an important role in promoting GC. Recent studies suggested GC is a heterogeneous disease; however, the detailed mechanisms that regulated GC are not fully understood. The mechanisms that control GC invasion and metastasis remain to be clarified [2].

Cancer stem cells (CSCs) constitute only a small percentage (0.05-1%) of tumor cells, but they may represent novel therapeutic targets for the treatment and prevention of tumor progression as they appear to be involved in cell migration, invasion, metastasis, and treatment resistance, all of which lead to poor clinical outcomes [3]. Therefore, CSCs are considered potential targets or drug carriers for the treatment of GC [4]. The identification of CSC-specific markers, the isolation and characterization of CSCs from malignant tissues, and the development of strategies for targeted eradication of CSCs represent an important opportunity in cancer research [5]. Our previous study has first demonstrated an independent subpopulation of gastric cancer cells expressing CD44 and CD90 in two individual cell lines (SNU-5 and SNU-16), which displayed specific biomarkers capable of identifying and isolating metastatic and tumorigenic CSCs.

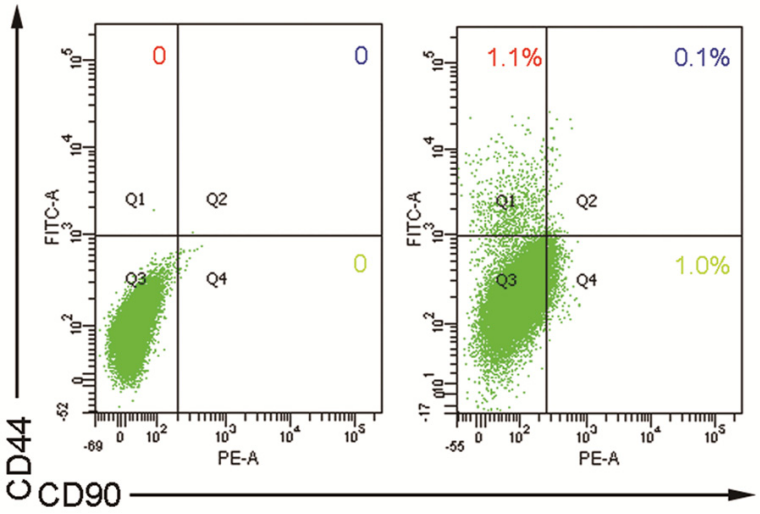

Figure 1. Cell subtype collection from SNU-5 cell lines by flow cytometry.

Furthermore, CD44+ CSCs were associated with tumor metastasis through EMT, whereas CD90+ CSCs were associated with high proliferation through cell cycle progression [6].

MicroRNAs (miRNAs) are reported to regulate the CSC characteristics by affecting signaling pathways and CSC signature genes. Particular miRNAs that are differentially expressed in CSCs or CSC-like cells in various tumors, are potential CSC markers. It is also known that miRNAs are a class of small, noncoding RNAs containing about 22 nucleotides that regulate gene expression. This attachment results in either mRNA degradation or inhibition of protein synthesis, making miRNAs useful tools in characterizing the role of select proteins in cell function [4]. Moreover, miRNAs have an important role in tumorigenesis, with altered expression in cancers associated with clinical outcome, therapy resistance, and tumor recurrence [7].

The purpose of present study is to find the miRNAs and their target mRNAs that could play special roles in different cell subtypes of GCs. In the study, CD44+ CD90- SUN-15 cell subtype was identified as CD44+ CSCs, CD44- CD90+ cell subtype was identified as CD90+ CSCs, and CD44- CD90- cell subtype was a control. Microarray analysis was used as an approach to identify differentially-expressed profiles of miRNAs and mRNAs in different cell subtypes. Then database was used to find out the specific expressed miRNA-mRNA pairs. Next we performed Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to investigate miRNA and mRNA associated with the phenotypes of CD44+ CSs and CD90+ CSCs.

Materials and methods

Cell culture and different cell subtypes' collection using flow cytometry

SNU-5 cells were acquired from the Chinese Academy of Science Cell Bank (Shanghai, China). SUN-5 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 1% penicillin/streptomycin (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cultures were maintained at 37°C in a humidification incubator with 5% CO_2.

In order to separate different cell subtypes, cultured SNU-5 cells were detached into single-cell suspensions using trypsin-EDTA, followed by staining of 1 × 10^6 cells in 500 μl phosphate-buffered saline (PBS)-0.5% bovine serum albumin (BSA) with fluorescence-labeled primary antibodies (1-5 μl), including CD90-phycoerythrin (1:10; eBioscience, San Diego, CA, USA) and CD44-fuorescein isothiocyanate (1:400; BD Biosciences, Franklin Lakes, NJ, USA) at 4°C for 60 min. Following washing, the labeled cells were analyzed and sorted immediately using a BD FACS AriaIII system (BD Biosciences). A blank control without labeling was analyzed to delineate the unstained populations. CD44+ CD90- SUN-15 cell subtype was identified as CD44+ CSCs, CD44- CD90+ cell subtype was identified as CD90+ CSCs and CD44- CD90- cell subtype was as control in our experiment. Those 3 subtypes were separately collected for extracting RNA. Two-color flow cytometry of the samples is shown in **Figure 1**.

RNA extraction

Total RNA was extracted from 3 different cell subtypes by soaking them in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance

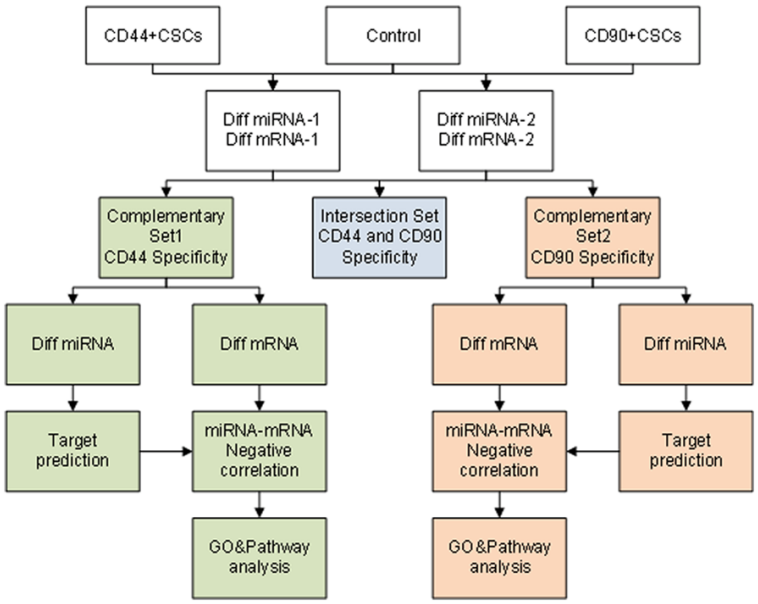

Figure 2. Flow chart for bioinformatic analysis.

with the manufacturer's instructions, which mainly includes homogenization, phase separation, RNA precipitation, RNA wash and RNA solubilization. RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

microRNA microarray analysis

RNA samples from different subtypes were analyzed using an Affymetrix GeneChip miRNA Array v. 4.0 (Affymetrix, Santa Clara, CA, U.S.). Total RNA was purified with mirVana™ miRNA Isolation Kit (AM1561). RNA quantity was measured using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA) labeled with Biotin using a 3DNA Array Detection FlashTag™ Biotin HSR kit (Genisphere, Hatfield, PA, U.S.) following the manufacturer's protocol, being subsequently hybridized overnight. The GeneChip® miRNA 4.0 arrays were washed and stained using the Affymetrix GeneChip Hybridization Wash and Stain Kit. They were then scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, U.S.) [8].

mRNA microarray analysis

mRNA Microarray analysis was performed by Captial-Bio Corporation (Beijing, China). Briefly, each purified sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, CapitalBio). The labeled cDNA was then hybridized onto the Agilent human lncRNA+mRNA Array V4.0 as indicated [9]. After washing the slides, the arrays were scanned on an Agilent Scanner by using the GeneSpring software V12.0 (Agilent Technologies).

Microarray data processing and analysis of differentially expressed miRNAs and mRNAs

To seek the specially expressed miRNAs and mRNAs in CD44+ CSCs and CD90+ CSCs, miRNAs and mRNA microarray data were analyzed and compared within each cell subtypes. To detect the differential expression and correlation of miRNAs and mRNAs in different cells, miRanda and Targetscan were selected to predict target genes of differentially expressed miRNAs. Only those miRNA-target pairs which were predicted by both algorithms can be selected out. For further analysis, the negative intersection of miRNA and mRNA was selected. The follow chart for bioinformatics analysis was depicted in **Figure 2**.

GO annotations and KEGG pathway analysis

Functional enrichment analysis was actualized according to the literature [Peng et al. 2016]. To understand the underlying pathways and biologic processes of specially expressed miRNAs and mRNAs in CD44+ CSCs and CD90+ CSCs, GO analysis was used to provide a controlled vocabulary to describe DE mRNAs ($P < 0.05$) in GO categories (http://www.geneontology.org). In addition, the KEGG database (http://www.genome.ad.jp/kegg/) was used to detect the potential functions of the changed target genes (mRNAs) in the identified pathways, with significance indicated by *P*-value < 0.05.

qRT-PCR validation

As previously described, total RNA was reverse transcribed into cDNA; then qRT-PCR was per-

Table 1. Primer sequences for reverse transcription quantitative polymerase chain reaction

	Primer sequences (5'-3')
H-ACTIN-S	CACCCAGCACAATGAAGATCAAGAT
H-ACTIN-A	CCAGTTTTTAAATCCTGAGTCAAGC
H-HMGA2-S	CACTTCAGCCCAGGGACAAC
H-HMGA2-A	TTTGCTGCCTTTGGGTCTTC
H-QKI-S	GCCTGATGCTGTGGGACCTAT
H-QKI-A	CTGCTTCAAGTTGTTTGGCTGTA
H-TRIM67-S	TGATGCCCTTGTGGATGCTT
H-TRIM67-A	GCAGCTTCAATGTGCAGTGATT
H-CASZ1-S	CCACAGATAAACCAGCCGTCAC
H-CASZ1-A	ACGTCCTGCTTACTCGTGAACCT
H-AMOT-S	CCGTTCTACCAATGAGGACCAA
H-AMOT-A	TGGATTCTCAGGGATTCCAGTT
H-USP31-S	AGCCTCCTCTGAAGCCACCAT
H-USP31-A	GCGACAGTCCCAGACAGAGGT
H-KALRN-S	AGCTACCAGTACGCCCTTGACC
H-KALRN-A	CGGTTGATGTTGACATAGGCATT
H-EPB41L4B-S	ACCTGCTTATGCTTTACACTTTCG
H-EPB41L4B-A	GGAATGAACCGAAACTCAGACAC
H-ATP2B2-S	GCGAATGCCAGCCTAGTCAA
H-ATP2B2-A	CACAGTGAAGTAGAGCACCAGGAT
H-EMC4-S	GCGACCCATTCAGGCACTTA
H-EMC4-A	AGACCCAAATACATAGGGACCAG
hsa-miR-3613-3p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCACGCGA
hsa-miR-3613-3p-S	ACACTCCAGCTGGGTTTGTTCGTTCGGCTC
hsa-miR-1910-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGCGGCA
hsa-miR-1910-5p-S	ACACTCCAGCTGGGCCAGTCCTGTGCCTG
hsa-miR-15b-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTAAACC
hsa-miR-15b-5p-S	ACACTCCAGCTGGGTAGCAGCACATCATGG
U6-RT	CTCGCTTCGGCAGCACA
U6-S	AACGCTTCACGAATTTGCGT

formed using an Applied Biosystems (Wilmington, DE, USA) 7500 RT-PCR system. GAPDH was used as an internal control to normalize mRNA expression levels [10]. miRNA expression levels were normalized using U6. The $2^{-\Delta\Delta CT}$ method was used for comparative quantitation. Three independent experiments were performed. The specific primers for each gene are listed in **Table 1**.

Statistical analysis

Statistical analysis was performed using SPSS V.16.0 (SPSS, Chicago, USA). The fold change and the Student's t-test were used to analyze the statistical significance of qRT-PCR results.

Results

Identification of differently expressed miRNAs in CD44+ CSCs and CD90+ CSCs

Through bioinformatics analysis, specially expressed miRNAs of CD44+ CSCs and CD90+ CSCs were identified with the Ratio > 2 or < 0.5. Compared with CD90+ CSCs and control, 31 miRNAs specially expressed in CD44+ CSCs were identified (**Figure 3A**). Of those identified differentially expressed miRNAs, 15 miRNAs were up-regulated and 16 miRNAs were down-regulated, which are listed in **Table 2**. Compared with CD44+ CSCs and control, 4 miRNAs specially expressed in CD90+ CSCs

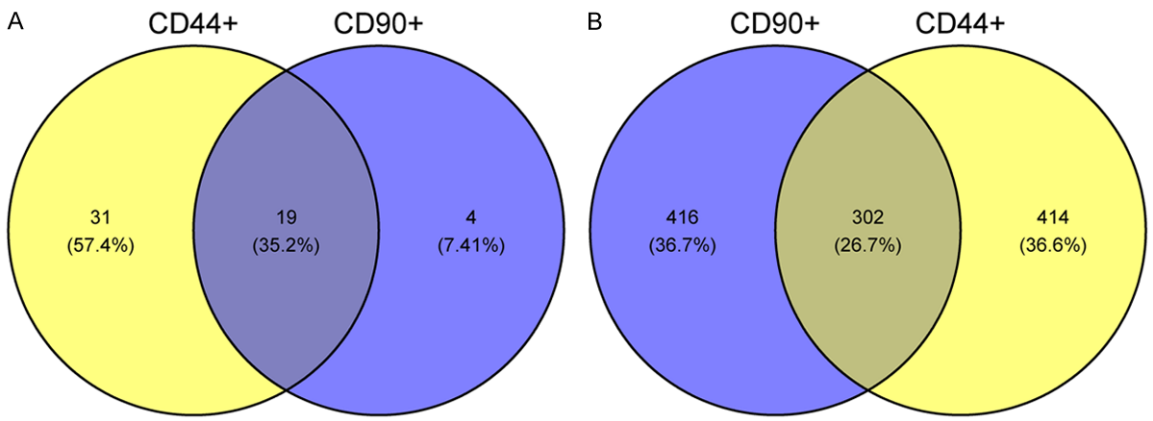

Figure 3. Analysis of aberrantly expressed genes in CD44+ CSCs and CD90+ CSCs. A. Venn diagram analysis of miRNAs. B. Venn diagram analysis of mRNAs.

(**Figure 3A**). All of those 4 miRNAs were up-regulated and listed in **Table 3**.

Identification of differently expressed mRNAs in CD44+ CSCs and CD90+ CSCs

Through bioinformatic analysis, specially expressed mRNAs of CD44+ CSCs and CD90+ CSCs were identified with the Fold change (abs) > 2. Comparing the expression of mRNAs in CD44+ CSCs with CD90+ CSCs and controls, 416 mRNAs specially expressed were identified (**Figure 3B**). Of those identified differentially expressed mRNAs, 148 mRNAs were up-regulated and 268 mRNAs were down-regulated, as listed in Supplemental Table 1. Comparing the expression of miRNAs in CD90+ CSCs with CD40+ CSCs and control, 414 mRNAs specially expressed in CD44+ CSCs were identified (**Figure 3B**). Of those identified differentially expressed miRNAs, 78 miRNAs were up-regulated and 336 miRNAs were down-regulated. Differentially expressed mRNAs are listed in Supplemental Table 2.

Negative correlations of differentially expressed miRNAs and mRNAs in CD44+ CSCs and CD90+ CSCs

In order to investigate the potential miRNA-mRNA pairs that play special roles in different cell subtypes, the correlation analysis of special expressed miRNAs and their negatively regulated target mRNAs were performed as indicated. Depending on the correlation analysis, 183 miRNA-mRNA pairs which were negatively correlated (FC(abs) > 2) were identified in CD44+ CSCs, of which there were 174 miRNA (up-regulation)-mRNA (down-regulated) pairs and 9 miRNA (down-regulation)-mRNA (up-regulated) pairs (**Figure 4A**). 12 miRNAs (up-regulation)-mRNA (down-regulated) negatively correlated pairs (FC(abs) > 2) were identified in CD90+ CSCs (**Figure 4B**).

Functional analysis of differentially expressed miRNA target mRNAs

In order to screen out the miRNA-mRNA pairs related to invasion and migration in CD44+ CSCs and proliferation in CD90+ CSCs, we conducted GO and KEGG pathway analysis. Records with *P*-value < 0.05 and enrichment > 2.0 in KEGG pathway were tallied. The results demonstrated that targeted differentially expressed mRNAs in CD44+ CSCs were most significantly enriched in the biological processes of tight junctions, transcriptional misregulation in cancer, salivary secretion, glycosaminoglycan biosynthesis-keratan sulfate, pancreatic secretion signal transduction, angiogenesis, insulin secretion, and cell cycle. Among these enriched signaling pathways, tight junction signaling pathways have been identified to contribute to invasion and migration (**Figure 5A**, **5B**). Therefore, we mainly focused on the study of differentially expressed mRNAs associated with tight junction pathways. In CD90+ CSCs, mucin type O-glycan biosynthesis, various types of N-glycan biosynthesis, N-Glycan biosynthesis, and aminoacyl-tRNA biosynthesis were the significantly en-

Table 2. Differential expression of miRNAs in CD44+ CSCs through bioinformatic analysis

Style	Transcript ID (Array Design)	Ratio	Sequence
up	hsa-miR-30a-5p	2.0332	UGUAAACAUCCUCGACUGGAAG
up	hsa-miR-30a-3p	2.5321	CUUUCAGUCGGAUGUUUGCAGC
up	hsa-miR-196a-5p	3.3402	UAGGUAGUUUCAUGUUGUUGGG
up	hsa-miR-30c-5p	2.3783	UGUAAACAUCCUACACUCUCAGC
up	hsa-miR-139-5p	2.9989	UCUACAGUGCACGUGUCUCCAGU
up	hsa-miR-15b-5p	2.1582	UAGCAGCACAUCAUGGUUUACA
up	hsa-miR-30b-5p	2.2861	UGUAAACAUCCUACACUCAGCU
up	hsa-miR-122-5p	2.9564	UGGAGUGUGACAAUGGUGUUUG
up	hsa-miR-125a-5p	2.1716	UCCCUGAGACCCUUUAACCUGUGA
up	hsa-miR-424-3p	2.357	CAAAACGUGAGGCGCUGCUAU
up	hsa-miR-181d-5p	2.5225	AACAUUCAUUGUUGUCGGUGGGU
up	hsa-miR-151b	2.1121	UCGAGGAGCUCACAGUCU
up	hsa-miR-4286	2.224	ACCCCACUCCUGGUACC
up	hsa-miR-3911	2.6467	UGUGUGGAUCCUGGAGGAGGCA
up	hsa-miR-4649-3p	2.1998	UCUGAGGCCUGCCUCUCCCCA
down	hsa-miR-668-5p	0.4999	UGCGCCUCGGGUGAGCAUG
down	hsa-miR-1185-1-3p	0.4964	AUAUACAGGGGGAGACUCUUAU
down	hsa-miR-4417	0.4722	GGUGGGCUUCCCGGAGGG
down	hsa-miR-4428	0.4704	CAAGGAGACGGGAACAUGGAGC
down	hsa-miR-4492	0.4673	GGGGCUGGGCGCGCGCC
down	hsa-miR-4665-5p	0.4455	CUGGGGGACGCGUGAGCGCGAGC
down	hsa-miR-4706	0.491	AGCGGGGAGGAAGUGGGCGCUGCUU
down	hsa-miR-4741	0.4806	CGGGCUGUCCGGAGGGGUCGGCU
down	hsa-miR-5739	0.4578	GCGGAGAGAGAAUGGGGAGC
down	hsa-miR-6126	0.3302	GUGAAGGCCCGGCGGAGA
down	hsa-miR-6732-5p	0.4213	UAGGGGGUGGCAGGCUGGCC
down	hsa-miR-6768-5p	0.4846	CACACAGGAAAAGCGGGGCCCUG
down	hsa-miR-6794-5p	0.3129	CAGGGGGACUGGGGGUGAGC
down	hsa-miR-7110-5p	0.3981	UGGGGGUGUGGGGAGAGAGAG
down	hsa-miR-7154-5p	0.4896	UUCAUGAACUGGGUCUAGCUUGG
down	hsa-miR-8073	0.4679	ACCUGGCAGCAGGGAGCGUCGU

Table 3. Differential expression of miRNAs in CD90+ CSCs through bioinformatics analysis

Style	Transcript ID (Array Design)	Ratio	Sequence
up	hsa-miR-1910-5p	3.2585	CCAGUCCUGUGCCUGCCGCCU
up	hsa-miR-378c	2.0804	ACUGGACUUGGAGUCAGAAGAGUGG
up	hsa-miR-3613-3p	3.2031	ACAAAAAAAAAAGCCCAACCCUUC
up	hsa-miR-4749-5p	2.0485	UGCGGGGACAGGCCAGGGCAUC

riched signaling pathways of targeted differentially expressed mRNAs (**Figure 5C**). There are no reports of those pathways related to proliferation.

qRT-PCR to validate miRNAs-mRNAs pairs in CD44+ and CD90+ CSCs

In CD44+ CSCs, six pairs of miRNAs-mRNAs were validated based on the above analysis. Gene AMOT, which was down-regulated and reported to suppress invasion, was selected. The regulated miRNA (hsa-miR-15b-5p) of AMOT and its target genes USP31, KALRN, EPB41L4B, ATP2B2, EMC4 were selected too. qRT-PCR results were consistent with the microarray data. As exhibited in **Figure 6A**, hsa-miR-15b-5p was significantly up-regulated ($P < 0.05$), and AMOT was significantly down-regulated ($P < 0.01$). USP31, KALRN, EPB41L4B, ATP2B2 and EMC4 were also downregulated.

In CD90+ CSCs, five pairs of miRNAs-mRNAs were validated based on the above analysis. The records with *P*-value < 0.05 and enrichment > 2.5 were selected. According to the standard, hsa-miR-3613-3p and its target genes QKI, TRIM67, CASZ1 were selected for variation. Hsa-miR-1910-5p and its target gene QKI and HMGA2 are selected too. qRT-PCR results were consistent with the microarray data, and demonstrated in **Figure 6B** that, compared with controls, hsa-miR-3631-3p and has-miR-1910-5p were significantly up-regulated ($P < 0.05$) in CD90+ CSCs. QKI, the target gene of hsa-miR-3613-3p and hsa-miR-1910-5p, was significantly down-regulated ($P < 0.01$). TRIM67, CASZ1 and HMGA2 were also downregulated.

Discussion

Recent advancements in the stem cell field confirm the importance of CSCs in the tumor

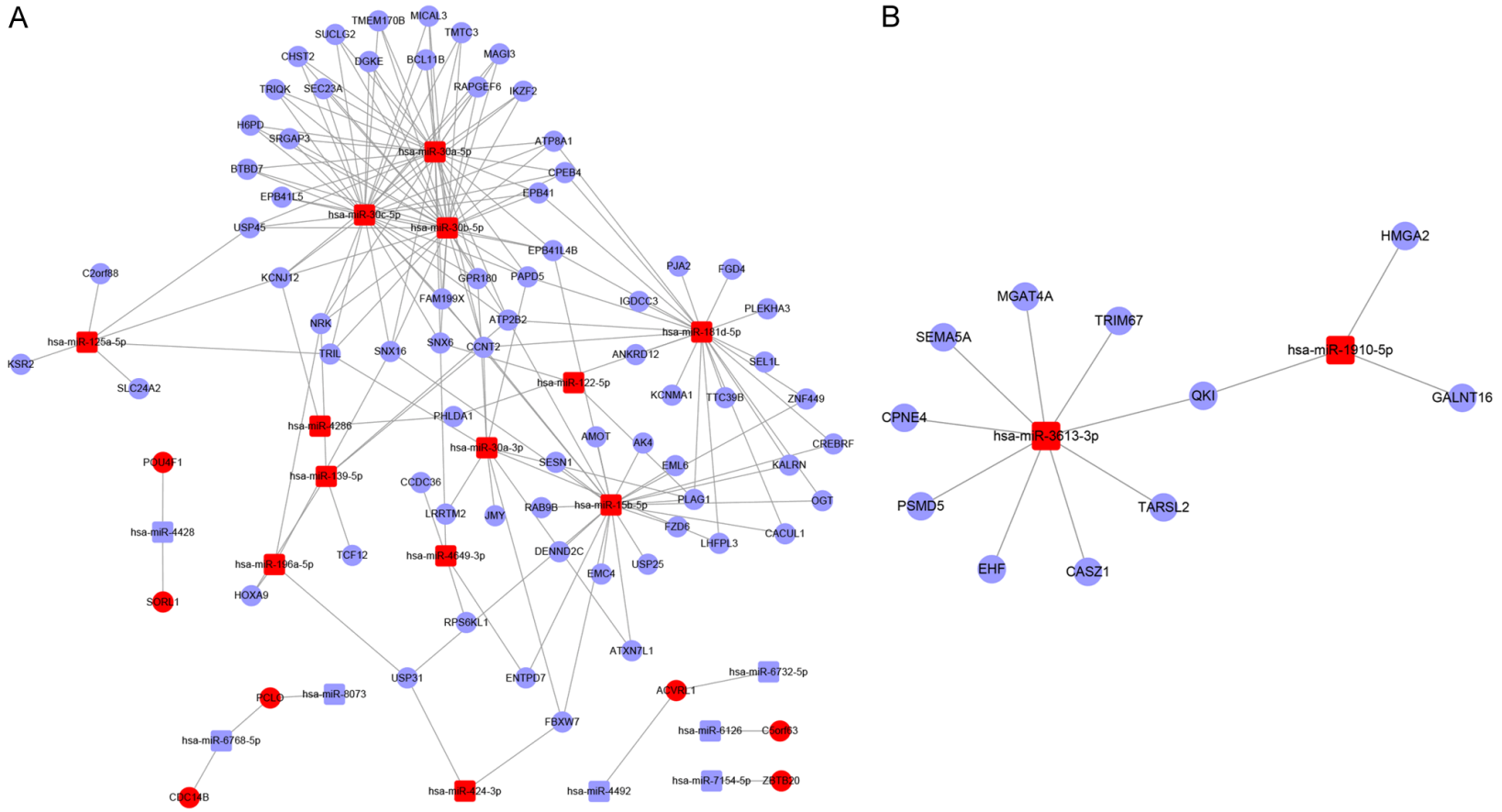

Figure 4. Network for negatively correlated expressed miRNA-mRNA pairs in CD44+ CSCs and CD90+ CSCs. A. Network of miRNA-mRNA pairs in CD44+ CSCs. B. Network of miRNA-mRNA pairs in CD90+ CSCs.

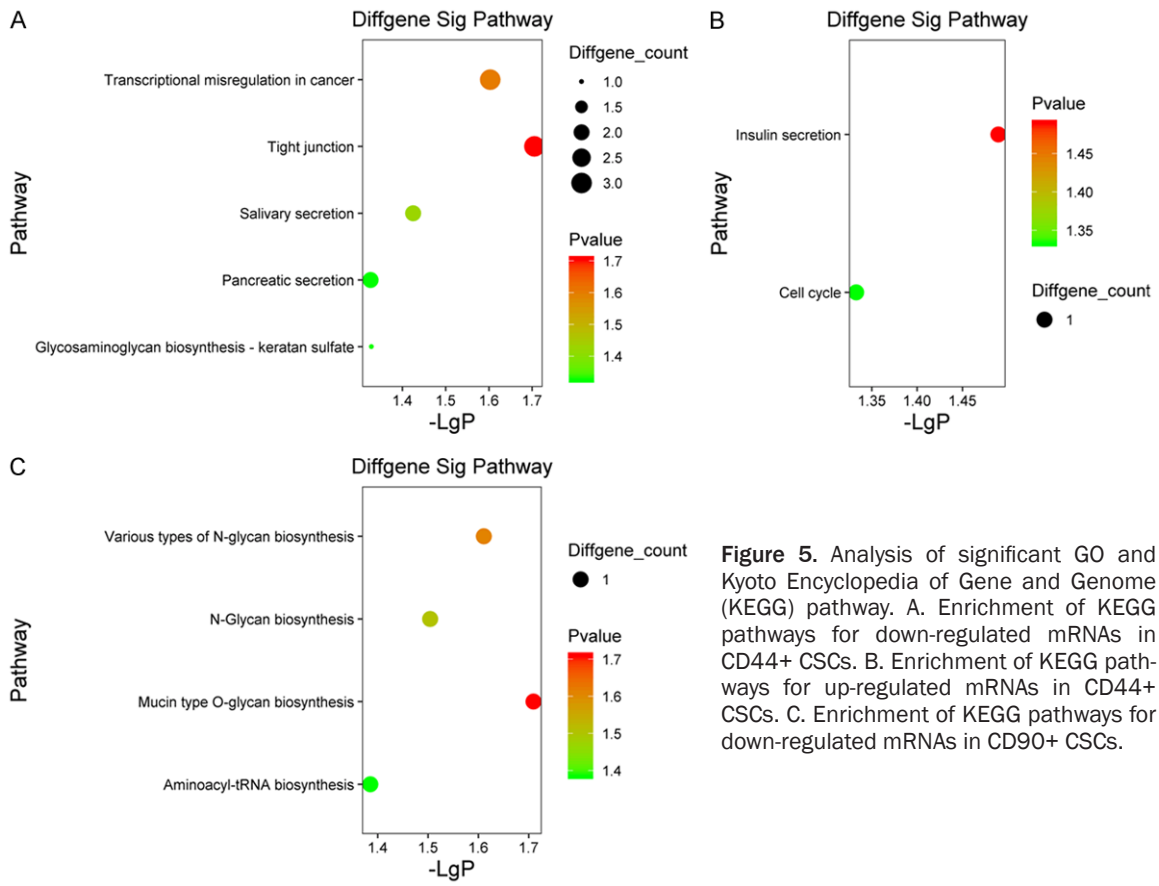

Figure 5. Analysis of significant GO and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway. A. Enrichment of KEGG pathways for down-regulated mRNAs in CD44+ CSCs. B. Enrichment of KEGG pathways for up-regulated mRNAs in CD44+ CSCs. C. Enrichment of KEGG pathways for down-regulated mRNAs in CD90+ CSCs.

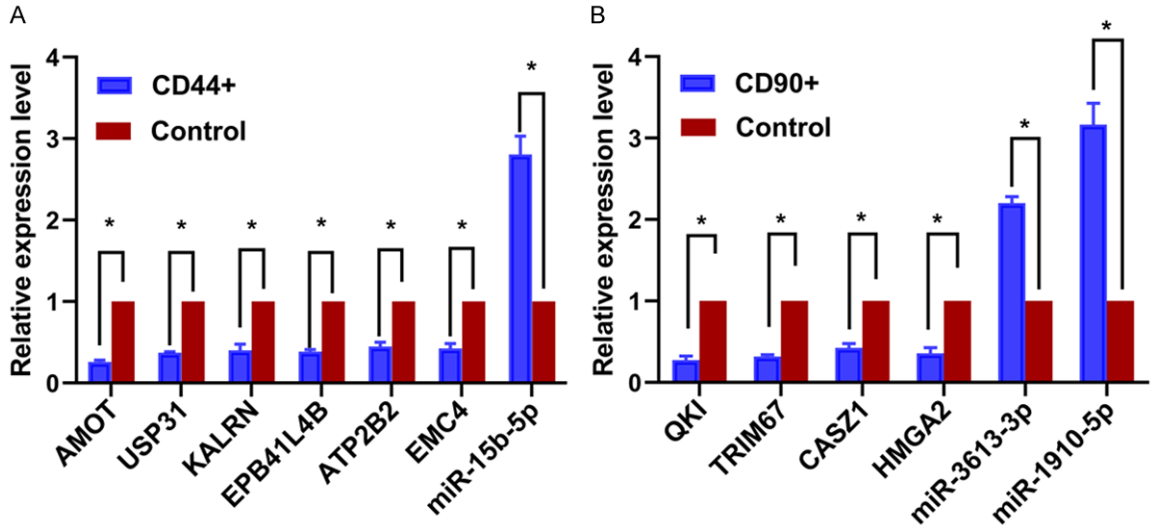

Figure 6. Validation of selected miRNA-mRNA pairs by qRT-PCR. A. qRT-PCR validation of expression of miRNA-mRNA pairs in CD44+ CSCs. B. qRT-PCR validation of expression of miRNA-mRNA pairs in CD90+ CSCs.

hierarchy and the origins of tumor initiation, heterogeneity, and propagation [11]. Our previous study showed that CD44+ and CD90+ CSCs obtained from the same gastric cancer cell lines exhibited higher tumorigenicity than CD44- CD90- cells. Our data also provide evi-

dence that CD44+ and CD90+ CSCs possess CSCs properties and are capable of generating heterogeneous tumors *in vivo* and tumor spheres *in vitro*, suggesting their potential as independent markers of gastric CSCs subpopulations [6]. miRNAs have been reported to regulate the CSC characteristics by affecting signaling pathways and CSC signature genes. The purpose of our experiment is to find out those particular miRNA-mRNA pairs differentially expressed in CD44+ and CD90+ CSCs, which are potential CSC markers.

Through bioinformatics analysis and comparing the expression of different miRNAs between CD44+, CD90+ and CD44- CD90- cells, specially expressed miRNAs and mRNAs of CD44+ CSCs and CD90+ CSCs were identified. Among those results, we found that the differential expression of some miRNAs was not consistent with their target mRNAs. Then the correlations of those key miRNAs and mRNAs distributions in different cell subtypes were further investigated. Depending on the correlation analysis, 183 negatively correlated miRNA-mRNA pairs were screened out in CD44+ CSCs. 17 miRNA-mRNA negatively correlated pairs were identified in CD90+ CSCs.

To better understand the biological functions and to find out the potential key miRNA-mRNA pairs associated with tumor metastasis of CD44+ CSCs and proliferation through cell cycle progression of CD90+ CSCs, we performed GO and KEGG pathway analysis. Among the KEGG pathways of CD44+ CSCs, tight junction signaling pathways mediate invasion and migration, which play vital roles in metastasis [12]. It is a down-regulated pathway. 3 key mRNAs are included, namely RAPGEF6, EPB41L4B and AMOT. RAPGEF6 was reported to induce activation of Rap1 and increase integrin-mediated cell adhesion and knockdown of it blocks EGFR-induced migration in human pancreatic carcinoma cells [13, 14]. EPB41L4B has been reported to be overexpressed in tumor cells with high migratory potential, such as metastatic melanoma and fibrosarcoma cells [15, 16]. Among those mRNAs, only AMOT is reported to inhibit endothelial cell migration and induces apoptosis in a cell type-specific manner [17]. Then we selected AMOT (down-regulated) and the associated miRNA hsa-miR-15b-5p (up-regulated) for further validation. Overexpression of miR-15b-5p has been previously reported in other malignancies, such as colorectal cancer, endometrial endometrioid adenocarcinoma, and non-small cell lung cancer [18, 19]. The other target genes of hsa-miR-15b-5p that were down-regulated for more than 2.5 folds were selected too. The target genes we selected for validation were AMOT, USP31, KALRN, ATP2B2, EMC4, EPB41L4B. The results from the bioinformatics analysis and qRT-PCR experiments were 100% in agreement.

It was found that suppression of the USP31 promotes sarcomagenesis by establishing the NF-κB pathway in undifferentiated pleomorphic sarcoma (UPS) [20]. There is no report of the association of KALRN with tumor, but KALRN has been reported to regulate neurite initiation, axonal growth, dendritic morphogenesis, and spine morphogenesis [21]. Elevated ATP2B2 gene expression was reported to be associated with larger tumors, higher grade, and nodal metastases [22]. There is no report of the association of ECM4 with tumor. Wang et al. showed that EMC6 has significant antitumor activity [23]. Then function of those selected genes indicated that those miRNA-mRNA pairs may play roles in regulating tumor metastasis in CD44+ CSCs; however, more research is needed for further functional validation.

Among the KEGG pathways of CD90+ CSCs, bioinformatics results did not show any gene associate with high proliferation. Genes were selected according to the record with *P*-value < 0.05 and enrichment > 2.5. Hsa-miR-3613-3p (up-regulated) and its target gene QKI (down-regulated), TRIM67 (down-regulated), and CASZ1 (down-regulated) were selected for variation. Hsa-miR-1910-5p (up-regulated) and its target gene QKI (down-regulated) and HMGA2 (down-regulated) were also selected. The results from the bioinformatics analysis and qRT-PCR experiments were 100% in agreement. Zhang et al. reported that hsa-miR-3613-3p affect the proliferation of HepG2 cells [24]. There are no reports showing that miR-1910-5p could regulate the proliferation of tumor cells. Zong et al. showed that QKI-5 inhibits the proliferation and transformation of lung cancer cells both in vitro and in vivo [25]. TRIM67 attenuates cell proliferation and induces morphologic

changes like neuronal differentiation in HEK-293T, HeLa, and N1E-115 cells [26]. CASZ1 gene was reported to suppress tumor and loss of CASZ1 may interrupt the normal developmental program of other tissues types and have a role in tumorigenesis of gastric intestinal type adenocarcinoma, head and neck squamous cell carcinoma, and clear cell renal cell carcinoma [27]. However, in previous studies, overexpression of HMGA2 promoted proliferation, migration, and invasion and inhibited apoptosis and cell cycle arrest in endometrial cancer cells [28]. Further research is needed to verify the roles of those miRNA-mRNA pairs in regulating proliferation in CD90+ CSCs.

In conclusion, novel miRNA-mRNA pairs of different cell subtypes were identified in our study. In CD44+ CSCs, hsa-miR-15b-5p was specially expressed and its down-regulated target genes AMOT, USP31, KALRN, EPB41L4B, ATP2B2 and EMC4 may act as important mediators of metastasis. In CD90+ CSCs, hsa-miR-3613-3p (up-regulated) and its target gene QKI (down-regulated), TRIM67 (down-regulated), CASZ1 (down-regulated), hsa-miR-1910-5p (up-regulated) and its target gene QKI (down-regulated) and HMGA2 (down-regulated) were screened out. These miRNA-mRNA pairs may be responsible for CD44-specific metastasis and CD90-specific proliferation respectively, and confirmatory studies are needed. The screened miRNA-mRNA pairs give us new insight for the mechanism of different phenotypes and biomarkers capable of identifying and isolating metastatic and tumorigenic CSCs. Interest in such miRNAs and their target genes needs further functional validation studies. Those miRNA-mRNA pairs may also act as potential treatment for GC.

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Disclosure of conflict of interest

None.

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