MicroRNA-182 downregulates metastasis suppressor 1 and contributes to metastasis of hepatocellular carcinoma

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Abstract

Background: miR-182 is one of the most significantly up-regulated miRNAs in hepatocellular carcinoma (HCC). Metastasis suppressor 1 (MTSS1), one target gene of miR-182, plays an important role in the metastasis of cancers. However, it remains unclear what role does function and mechanism of miR-182 and MTSS1 play in HCC.

Methods: miR-182 expression was tested in 86 cases of paired HCC and normal tissues by real-time PCR and the relationships between miR-182 expression and clinicopathological parameters were analyzed. The expression of MTSS1 was evaluated by immunohistochemistry and western blot in the above tissues and its correlation with miR-182 expression was analyzed. Moreover, western blot and invasion assays were performed after transfection of pre-miR-182 or anti-miR-182 to HCC cell lines. In addition, luciferase assays was performed to confirm the regulation of miR-182 on MTSS1.

Results: Compared with normal tissue, miR-182 was up-regulated and MTSS1 was down-regulated in HCC tissues. Moreover, the over-expression of miR-182 was correlated with intrahepatic metastasis (p = 0.034) and poor prognosis (p = 0.039) of HCC patients. There was a negative correlation between miR-182 and MTSS1 expression in both HCC tissues (r = −0.673, p < 0.01) and HCC cell lines (r = −0.931, p = 0.021). Furthermore, the up-regulation of miR-182 resulted in the down-regulation of MTSS1 and increased invasive potential of HUH-1, and reverse results were also confirmed when the expression of miR-182 was inhibited. In addition, the results of the luciferase assay demonstrated the targeted regulation of miR-182 on MTSS1.

Conclusions: miR-182 could promote metastasis of HCC and inhibit the expression of MTSS1. miR-182 and MTSS1 are potential prognostic markers and/or therapeutic targets in HCC.

Keywords: Hepatocellular carcinoma, miR-182, Metastasis suppressor 1, Metastasis

Background

Hepatocellular carcinoma (HCC), one of the most notoriously invasive cancers, is among the top 10 most prevalent cancers worldwide, accounting for ~600,000 deaths annually [1,2]. At present, surgical resection/liver transplantation is the only treatment modality to confer survival benefit in HCC patients, and the overall 5-year survival rate for HCC patients is less than 5% [3]. The most important reason leading to poor prognosis is intra hepatic metastasis [4]. It is thus necessary to elucidate the molecular mechanisms underlying HCC metastasis and identify novel therapeutic targets.

Recently, it has been manifested that the deregulation or dysfunction of miRNAs is involved in cancer development and related to clinical outcomes of cancer patients including HCC [5-12]. Yu, et al reported miR-182 was one of the most significantly up-regulated miRNA in HCC patients [13]. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor [14,15], which indicates that miR-182 may promote the metastasis of HCC through targeting on some genes. In both websites Target scan and PicTar, we found hundreds of target genes regulated by miR-182. Among those genes with highly conserved binding sites, metastasis suppressor 1

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MiR-182 analysis demonstrated the negative correlation between 182 neoplastic tissues were collected and stored at observed in 46.5% (40/86) patients. HCC and non-guided biopsy to confirm the diagnosis. Recurrence was computed tomography (CT) scan, if necessary, by ultrasound-further evaluations were performed by abdominal com- mean time of follow-up was 28.3 months. The percentage of AFP (>100 ng/ml) was 80.2%. Moreover, one tumor was detected in 79.1% (68/86) patients and multiple tumors (≥2) were found in 20.9% (18/86) patients with totally 29 metastatic lesions. The average tumor size was 5.8 ± 2.7 cm (0.4-16 cm). Histologically, 32.6% (28/86), 46.5% (40/86) and 20.9% (18/86) tumors were grade 1, 2 and 3, respectively. No chemotherapy was performed after radical resection. Patients were followed-up at the outpatient clinic with measurement of the serum alpha-fetoprotein level and hepatic ultrasonography every 2–4 months from the date of initial treatment. The mean time of follow-up was 28.3 months (range 3–56 months). When recurrence was suspected, further evaluations were performed by abdominal computed tomography (CT) scan, if necessary, by ultrasound-guided biopsy to confirm the diagnosis. Recurrence was observed in 46.5% (40/86) patients. HCC and non-neoplastic tissues were collected and stored at −80°C until analysis. For every frozen tumor tissue, we cut frozen slide and did HE staining and evaluated the percentage of tumor cells. The percentage of tumor cells was about 90%. In addition, paraffin-embedded HCC tissues were also collected.

**RNA extraction and quantitative RT-PCR for miR-182**

Total RNA, including miRNA, was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA was reversely transcribed using the corresponding RT Primer and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The expression of miR-182 and its control RNU44 were detected using TaqMan miRNA assay system (Applied Biosystems, Foster City, CA, USA). The median miRNA intensity value of 86 patient samples was used as the threshold, and patients were divided into two groups (below median, group low miR-182 and above median, group high miR-182) according to the expression of miR-182.

**Immunohistochemistry staining and evaluation for MTSS1**

Immunohistochemistry (IHC) was used to detect MTSS1 expression in paraffin-embedded HCC tissues. Five-μm sections of paraffin-embedded HCC tissue were baked at 65°C for 2 h, followed by deparaffinization using standard procedures. After antigen retrieval, MTSS1 antibody (Cell Signaling Technology, Inc. Danvers, MA, USA) was applied to slides, followed by the secondary antibody conjugated with horseradish peroxidase. Signals were revealed by using the Histostain Plus kit (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instruction. 3, 3-Diaminobenzidine (DAB) was used as a chromogen. The sections were counter-stained with hematoxylin. We prepared a negative control by substituting PBS for the antibody. MTSS1 protein expression was evaluated by two pathologists. MTSS1-positive samples were defined as those with brown staining in the cytoplasm. The results of MTSS1 immunohistochemical analysis were estimated with semi-quantity method. The staining intensity was graded on a scale from 0 to 3 (0 for no staining, 1 for weak immunoreactivity, 2 for moderate immunoreactivity, and 3 for strong immunoreactivity) The percentage of immunoreactivity was scored on a scale from 0 to 4 (0, no positive cells; 1, <25% of cells positive; 2, 25%–50% of cells positive; 3, 50–75% of cells positive; and 4, >75% cells positive). Finally, a total score (negative: 0; weak: 1–2; medium: 3–5; strong: 6–7) was obtained by adding the scores of staining intensity and percentage positivity.

**Western blot for MTSS1**

Cell lysates were harvested with 2% sodium dodecyl sulfate (SDS)-125 mM Tris/HCl (pH 7.4). Cell lysates (25–30 ug of protein) were resolved in Tris/glycine SDS/PAGE gels and transferred to PVDF membranes. Membranes
were probed with primary antibodies overnight at 4°C and incubated with horseradish-peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The background was subtracted, and the signals of the detected bands were normalized to the amount of loading control β-actin (Cell Signaling Technology, Inc. Danvers, MA, USA) band. The protein levels were quantified using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA. http://rsb.info.nih.gov/ij).

**Cell culture and transfection**

Human HCC cell lines HLE, HLF, HepG2, Hep3B and HUH-1 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Invitrogen) except HepG2 (MEM) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) at 37°C in a humidified incubator containing 5% CO2.

For transfection, 2 × 10^5 HLF or HUH-1 cells were seeded into each well of a 6-well plate and incubated overnight, then the cells were transfected with Pre-miR miRNA Precursor Molecule pre-182 (pre-miR-182) and anti-miR miRNA inhibitor anti-182 (anti-miR-182) (Applied Biosystems) at a final concentration of 100 nM using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The specificity of the transfection was verified using the Pre-miR miRNA Precursor Molecule Negative Control #1 (control pre-miR) and Anti-miR miRNA Inhibitors Negative Control #1 (control anti-miR) (Applied Biosystems). The expression levels of miR-182 and MTSS1 were quantified 24 h after transfection, and the cells were used for western blot analysis.

**3’ UTR luciferase reporter assay**

The human MTSS1 3’ UTR luciferase reporter construct (MTSS1-3’UTR WT) was generated by cloning MTSS1 mRNA 3’UTR sequence into downstream of pMIR-Report construct (Ambion, Foster City, CA, USA). The MTSS1 3’ UTR sequence was generated by PCR using primer MTSS1 3’UTR F Spel: 5’-AAACTAGTGGATT TTTCGAGGGT GCCAAATTCCATTAAA-3’ and primer MTSS1 3’UTR R SacI: 5’-GGAGGCTCATTGGCACA CATTATTTATTTCA-3’. The miR-182 target site-mutation MTSS1 3’ UTR luciferase reporter 1 (MTSS1-3’UTR mutation 1) construct was generated by employing direct-site mutagenesis using mutation primers which mutate the miR-182 binding site from TCTGAAGGTCGGTA. miR-182 target site-mutation MTSS1 3’ UTR luciferase reporter construct was mutated from TTGCCAA to TTAAGGTCGGTA.

HUH-1 cells were co-transfected with miR-182 plasmid and wild-type or mutant MTSS1 3’ UTR luciferase reporter construct and luciferase activities were measured using the Dual-Glo Luciferase. Data were normalized by dividing Firefly luciferase activity with that of Renilla luciferase.

**In-vitro invasion assays**

HLF and HUH-1 cell invasion assays were performed using 24-well Matrigel Invasion Chambers (BD Biosciences, CA, USA). The lower chambers were filled with 0.75 ml of DMEM medium containing 10% fetal bovine serum (FBS). A cell suspension of 2 × 10^5 in 0.5 ml DMEM medium was added into each well of the upper chamber. After the cells were incubated for 24 h at 37°C in a humidified incubator with 5% CO2, the invasive cells attached to the lower surface of the membrane insert were fixed in 10% formalin at room temperature for 5 min and stained with 0.05% crystal violet. The non-invading cells that remained on the upper surface of the membrane were removed by scraping. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

**Statistical analysis**

Differences in MTSS1 immunohistochemical staining between groups were compared using chi-square or Fisher exact tests in human samples. The correlation between MTSS1 expression and miR-182 was evaluated by calculating the Spearman rank correlation coefficient. Moreover, mean ± SD of clinicopathological variables were calculated, and differences in the means were analyzed using one-way analysis of variance or Student’s t test. We also used the Cox proportional hazards regression model in our multivariate analysis. SPSS version 16.0 (IBM) was used to perform our statistical analysis. Two-tailed P values < 0.05 were considered statistically significant.

**Results**

The expression of miR-182 and its correlation with clinical-pathological features

To investigate the role of miR-182 in HCC development, we tested the expression of miR-182 in 86 HCC and matched non-neoplastic tissues (Figure 1A). The relative expression of miR-182 in HCC samples (2.21 ± 1.29) was significantly higher than that of matched normal tissues (1.12 ± 0.47) (p < 0.01) (Figure 1B). Hence, we considered the up-regulation of miR-182 may contribute to HCC tumorigenesis. Furthermore, the relative level of miR-182 in poorly differentiated HCC (3.28 ± 1.79) was almost one time higher than that in well (1.62 ± 0.68) and medium differentiated cases (2.14 ± 0.83) (Figure 1B).
which suggested that miR-182 might also correlate with the progress of HCC.

For better understanding the potential role of miR-182 in HCC progression, we analyzed its correlation with some clinicopathological variables including age, sex, HBV infection, AFP, tumor number, tumor size, expression of MTSS1, histological grade, portal vein invasion and recurrent time (Table 1). Based on the median value (1.92) of miR-182 expression, all patients were divided into two groups including group with low expression of miR-182 and group with high expression of miR-182.

Intra-hepatic metastasis (tumor number ≥ 2, \( p = 0.034 \)) and higher recurrence (\( p = 0.031 \)) tended to occur in the patients with high expression of miR-182. Though the \( p \) values did not reach statistical significance, the patients with high expression of miR-182 had a tendency to undergo occur portal vein invasion (\( p = 0.096 \)) (Table 1).

The recurrent percentage is 46.5% (40/86) for all patients. The median disease-free survival time in group with low miR-182 and group with high miR-182 was 27.0 months and 24.0 months, respectively. The Kaplan–Meier method revealed that higher miR-182 expression level correlated with significantly reduced disease-free survival (42.0 ± 2.93 months in group with low miR-182 versus 31.2 ± 2.79 months in group with high miR-182, \( p = 0.039 \)) (Figure 2). Multivariate survival analysis revealed that multiple tumors (\( p = 0.023 \)) and high expression of miR-182 (\( p = 0.022 \)) were significantly correlated with the poor prognosis of HCC patients (Table 2). The result further indicated the importance of miR-182 up-regulation in HCC development.

Table 1 The relationships between miR-182 expression and clinicopathologic features

| Variables                          | Low miR-182 expression (n = 43) | High miR-182 expression (n = 43) | \( p \)  |
|-----------------------------------|---------------------------------|---------------------------------|---------|
| Age (≤51 years vs ≥51 years)      | 21/22                           | 25/18                           | 0.387   |
| Gender (male vs female)           | 35/8                            | 32/11                           | 0.436   |
| HBV (positive vs negative)        | 37/6                            | 35/8                            | 0.341   |
| AFP (<100 ng/ml vs ≥100 ng/ml)    | 10/33                           | 7/36                            | 0.417   |
| Tumor number (n < 2 vs n ≥ 2)     | 38/5                            | 30/13                           | 0.034   |
| Tumor size (<5 cm vs ≥5 cm)       | 16/27                           | 19/24                           | 0.510   |
| MTSS1 (positive vs negative)      | 21/22                           | 16/27                           | 0.115   |
| Tumor grade (1 vs 2, 3)           | 16/27                           | 12/31                           | 0.174   |
| Portal vein invasion (no vs yes)  | 34/9                            | 27/16                           | 0.096   |
| Recurrent (no vs yes)             | 28/15                           | 18/25                           | 0.031   |

**AFP**: α-fetoprotein.

The expression of MTSS1 is down-regulated and negatively correlated with miR-182 in HCC

**MTSS1** protein expression was tested with IHC in HCC and paired normal tissues (for some cases, there are tumor and adjacent normal tissue in the same slide). **MTSS1** was positive in the cytoplasm of tumor and normal liver cells. **MTSS1** was often highly expressed in normal tissue (Figure 3 A, B and E), while drastically reduced **MTSS1** expression was shown in the tumor

![Figure 1](http://www.biomedcentral.com/1471-2407/12/227)
cells (Figure 3 B, D and E). Totally, MTSS1 was positive in 79% (68/86) of normal tissue and in 43% (37/86) of HCCs (43%). The rate of MTSS1 positive case in HCC was significantly lower than that in paired normal tissue ($p < 0.001$, Figure 3G). Among the 37 cases with MTSS1 positive expression, 13 (35%), 17 (46%) and 7 (19%) cases showed weak, moderate and strong expression of MTSS1, respectively. Moreover, no difference of MTSS1 expression was found among the multiple lesions in the same patient. The MTSS1 positive rate in metastatic HCC (17%, 3/18) was significantly lower than that in non-metastatic HCC (50%, 34/68) ($p < 0.001$, Figure 3G).

In addition, the tumor thrombus in small hepatic vein also showed low expression of MTSS1 (Figure 3 F).

For the MTSS1 positive cases tested by IHC, the examination of Western Blot were further performed and the expressions were quantified (Figure 4 A). Negative correlation between the expression of miR-182 and that of MTSS1 in HCC was indicated in Figure 4 B ($r = -0.673; p < 0.01$), which suggested MTSS1 maybe one important functional protein contributing to the oncogenic role of miR-182. Meanwhile, the negative correlation between miR-182 and MTSS1 expression were also found in HCC cell lines ($r = -0.931; p = 0.021$) (Figure 4 C and D).

miR-182 promotes invasion and inhibits MTSS1

Next, we sought to investigate the molecular mechanism responsible for the oncogene effect of miR-182 on HCC observed above. As miRNAs function mainly through inhibiting their target mRNAs by binding to the 3' UTR, we searched the putative target genes of miR-182 in Target Scan and Pictar. In both websites, 841 and 702 conserved targets were found, respectively. Among those targets, human MTSS1, known to have critical roles in the inhibition of cancer metastasis, contained two putative conserved miR-182 binding sites with high context scores (Figure 5A). To verify whether MTSS1 was a direct target of miR-182, a dual-luciferase reporter system was used by co-transfection of miR-182 and a luciferase reporter plasmid containing the 3' UTR of human MTSS1 into HUH-1. As shown in Figure 5B, the luciferase activity was significantly inhibited by miR-182 co-transfection, mutation either of the two miR-182 binding site, while miR-182 failed to inhibit the expression of luciferase construct with both binding sites mutated, suggesting that miR-182 could directly target on the 3' UTR of MTSS1.

As one target gene of miR-182 demonstrated above, the expression of MTSS1 was down-regulated in HUH-1 with transfected miR-182 and up-regulated in HLF with transfected anti-miR-182 (Figure 6 A). An in vitro
invasion assay indicated that the relative invasiveness of HLF transfected with anti-miR-182 was specifically reduced by approximately 41% \((p < 0.05)\) and the relative invasiveness cells of HUH-1 transfected with miR-182 was increased by approximately 36% \((p < 0.05)\) (Figure 6B). The result in vitro further demonstrated that miR-182 could promote metastasis of HCC and inhibited the expression of MTSS1.

**Discussions**

Up-regulation of miR-182 was suggested to exist in a large part of HCC tissues [15]. In our HCC cases with complete clinical data, we also found the up-regulation of miR-182 and its up-regulation was significantly associated with intrahepatic metastasis (tumor number \(\geq 2\)) and early recurrence, which is an important clinical determinant for the prognosis of HCC patients. Up-regulation of miR-182 was further suggested to correlate with reduced disease-free survival of HCC patients. Hence, determination of miR-182 expression level in HCC tissues may be a novel approach to predict and identify the prognosis of HCC patients.

Although miRNA profile did reveal very prospective features in cancer, the functions and real targets of
miRNAs were largely unknown. The predicted targets of the majority of microRNAs based on sequence homology remained to be comprehensively validated by *in vitro* and *in vivo* experiments. Target scan and Pictar showed that metastasis suppressor 1 (*MTSS1*) is one important target of *miR-182* with a high context score. Meanwhile, we found its expression in HCC decreased significantly compared to that of adjacent normal tissue and negatively correlated with *miR-182* expression in HCC cell lines. N: normal tissue; T: tumor.

Figure 4 Correlation between *miR-182* and *MTSS1* expression in HCC tissues and cell lines. **A:** Relative expression of *miR-182* was detected by real time RT-PCR and *MTSS1* expression was tested with Western blot and quantified with ImageJ software in 5 paired HCC and normal tissues. **B:** There was a negative correlation between *miR-182* and *MTSS1* expression (n = 37). **C:** The expression of *miR-182* is high and *MTSS1* expression is low in HLF and HLE, whereas Hep-G2 and HUH-1 showed low expression of *miR-182* and high expression of *MTSS1*. **D:** The expression of *MTSS1* was negatively correlated with *miR-182* expression in HCC cell lines.

Figure 5 *miR-182* directly targets human *MTSS1*. **A:** 3' UTR region of *MTSS1* mRNA is partially complementary to *miR-182*. Target-Scan and Pictar analysis revealed two *miR-182* binding sites in *MTSS1* UTR sequence. **B:** Effect of *miR-182* on the luciferase activity of Luc-*MTSS1*-3'UTR and Luc-*MTSS1*-3'UTR mutation. The assay was done in HUH-1 cells as described in Materials and Methods. Renilla and firefly luciferase activities were measured with the Dual-Luciferase Reporter system (Promega) 24 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase expression for each sample. Each experiment was performed in triplicate. Data are shown as mean ± s.d. *p < 0.01.
correlated with the expression of miR-182, which indicated MTSS1 may be the regulation target of miR-182.

MTSS1, also known as MIM (missing in metastasis), was originally identified by Lee et al. [18] as a potential metastasis suppressor gene that was present in non-metastatic bladder cancer cell lines, but was not expressed in a metastatic bladder cancer cell line [19]. This gene, mapped to human chromosome 8q24.1, encodes a 5.3 kb mRNA and a polypeptide predicted to be an actin-binding protein of 356 amino acids with homology to the WASp (Wiscott-Aldrich Syndrome protein) family [20]. Functional analyses of MTSS1 have shown that MTSS1 induced actin-rich protrusions resembling microspikes and lamellipodia at the plasma membrane and promoted disassembly of actin stress fibres [21]. Actin filament assembly is associated with cytoskeletal structure organization and many forms of cell motility [22]. These data have suggested that MTSS1 protein may be important in regulating cytoskeletal dynamics, and as a consequence it would play a potential role in the invasion and metastatic behavior of cancer cells. Therefore, the down-regulation of MTSS1 potentiated by the up-regulation of miR-182 may further aggravate the epigenetic changes in HCC. We then focused on the mechanisms that whether the up-regulation of miR-182 mediates the inhibition of MTSS1 and induced epigenetic alterations in HCC pathogenesis.

miR-182 can bind to MTSS1 at two conserved sites with a high context score. Our luciferase assay in HCC cell lines demonstrated MTSS1 can be regulated directly by miR-182. The interesting results in HCC cell lines is that cells with high invasive ability showed higher expression level of miR-182 than those with low invasive potential, which is inversely related with the expression of MTSS1. Analyses on human samples reinforced the relevance of miR-182 regulation on MTSS1 in HCC by revealing an inverse correlation between their expressions. Considering the characteristic heterogeneity of HCC and that MTSS1 is regulated by additional mechanisms, a statistically significant association with miR-182 is especially remarkable. The ability of MTSS1 over-expression to counteract miR-182’s pro-invasion effects unequivocally shows the importance of this inverse relationship in HCC metastasis. The functional analysis of miR-182 together with MTSS1 in animal models will particularly further evaluate their metastatic role and show us the clinical treatment value for patients with HCC. That would be our future research aim.

Concerning the target of miR-182, Miguel and et al. also reported that the microRNA promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor [13]. Together with our study, it is consistent with current opinions that a single miRNA can target multiple mRNAs, named ‘targetome’, to post-transcriptionally regulate gene expression [23]. Hence, it is probable that we are still far from unveiling the last target of miR-182. According to this presumption,
interesting future work may be carried out to identify the ‘targetome’ and the entire roles of miR-182 in cancer development. Another important issue is why miR-182 is up-regulated in HCC and other cancers [15,24]. The current view suggests that miRNA expression is mainly controlled at the transcriptional level. A large number of transcription regulators that influence the transcription and production of miRNAs have been identified including Myc, E2F, p53, and STAT3 [25-27]. Another possible mechanism for the up-regulation of miRNAs in cancer may result from the amplification of DNA copy number. Such as miR-182 is one member of a miRNA cluster in a chromosomal locus (7q31-34) frequently amplified in HCC [13], the amplification may cause the up-regulation of miR-182. This is our future research field.

Conclusions

Our study suggests a model of tumor progression in which elevated miR-182 expression and subsequent down-regulation of MTSS1 promotes aggressiveness of HCC. These results suggest that miR-182 and its downstream effectors could prove to be useful prognostic markers and/or therapeutic targets in HCC.

Competing interests

All authors declare that they have no competing interests.

Authors’ contributions

JW together with JL conceived of the study, and participated in its design and coordination and helped to draft the manuscript. JS carried out the molecular biological studies and drafted the manuscript. CW collected all the samples. JW, JL, and CW participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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