Original Article
MiR-330-3p inhibits gastric cancer progression through targeting MSI1

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Abstract: Increasing evidences demonstrated that microRNAs (miRNAs) play critical roles in the human tumor development and progression. In our study, we found that miR-330-3p expression was downregulated in gastric cancer cell lines and tissues. Ectopic expression of miR-330-3p suppressed the gastric cancer cell proliferation, colony formation and migration. Overexpression of miR-330-3p promoted E-cadherin expression and inhibited the expression of N-cadherin, vimentin and snail. We identified Musashi-1 (MSI1) as a direct target gene of miR-330-3p in gastric cancer cell. In addition, MSI1 was upregulated in gastric cancer cell lines and tissues and the MSI1 expression was inversely correlated with miR-330-3p expression in gastric cancer tissues. MiR-330-3p expression was increased in gastric cancer cells after treated with histone deacetylase inhibitor trichostatin A (TSA) and DNA methylation inhibitor 5-aza-CdR (AZA). These indicated that downregulated expression of miR-330-3p was partly mediated by gene promoter region hypermethylation. These results suggested that miR-330-3p acted as a tumor suppressor gene in GC.

Keywords: Gastric cancer, microRNAs, miRNAs, miR-330-3p, Musashi-1 (MSI1)

Introduction

Gastric cancer (GC) is the second most common cause of cancer related death and the fourth most common cancer worldwide [1-4]. It is estimated that about one million new GC cases are diagnosed per year [5-7]. Although advances in chemotherapy and surgery have been achieved, the prognosis of GC is still poor with the 5-year overall survival rate below 30% [8-10]. The main reason is that most GC patients are diagnosed at advanced stage [11-13]. Therefore, identification of new biomarkers and therapeutic targets are pivotal for improving GC prognosis.

MicroRNAs (miRNAs) are small, endogenous and noncoding RNAs that regulate gene expression through decreasing the stability and therefore repressing the translation of mRNAs [14-17]. Deregulation of miRNAs is found in many tumors such as gallbladder cancer, osteosarcoma, cutaneous squamous cell carcinoma, bladder cancer, ovarian carcinoma and breast cancer [18-22]. Increasing evidences have demonstrated that miRNAs play an important role in many biological processes such as cell development, proliferation, apoptosis, migration, invasion and differentiation [23-30]. Therefore, it is crucial to study the roles of miRNAs in the development of GC [31, 32].

In this study, we showed that miR-330-3p expression was downregulated in gastric cancer cell lines and tissues. Ectopic expression of miR-330-3p suppressed the HGC-27 cell proliferation, colony formation and migration. Musashi-1 (MSI1) was identified as a direct target gene of miR-330-3p in the GC cell.

Materials and methods

Tissue and cell lines cultured and cell transfection

Human GC tissues and adjacent normal tissues were surgically collected from GC patients in our hospital. This study was proved by the Ethics Review Board of the Affiliated Yan An Hospital of Kunming Medical University and
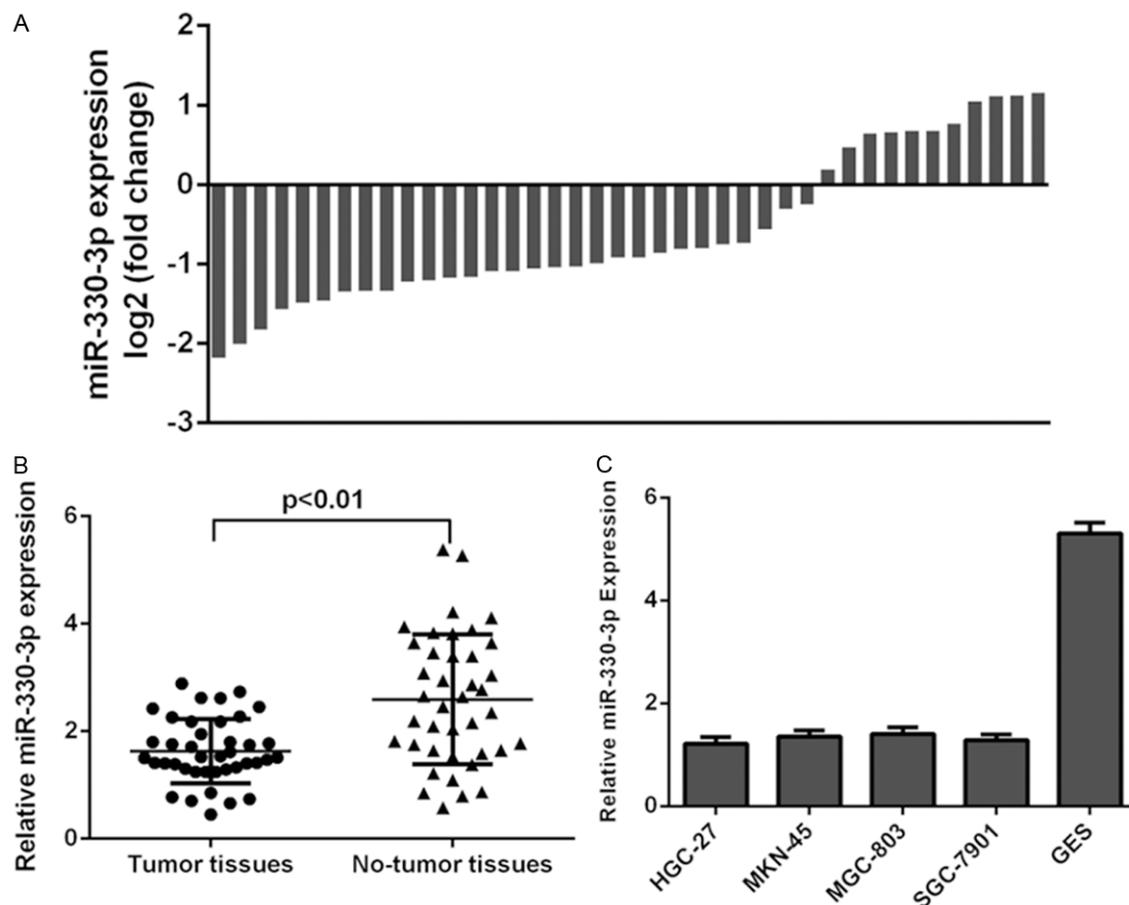
Figure 1. miR-330-3p was downregulated in gastric cancer cell lines and tissues. A: Of the 40 samples, miR-330-3p expression was downregulated in the 29 patients (29/40, 72.5%) compared to adjacent tissues. B: The expression of miR-330-3p was downregulated in the GC tissues compared with adjacent no-tumor tissues. C: miR-330-3p in the GC cell lines (MGC-803, MNK-45, SGC-7901 and HGC-27) and normal gastric mucosa cell line (GES) was measured by using qRT-PCR.

written informed consent was collected from each patient. GC cell lines (MGC-803, AGS, SGC-7901, HGC-27) and one normal gastric mucosa cell line (GES) were collected from the Chinese Academy of Science and cultured in the RPMI-1640 medium (Life Technologies, USA) contained with penicillin and streptomycin. miR-330-3p mimic and scramble oligonucleotide were collected from GenePharma (Shanghai, China). Cell transfection was performed using the Lipofectamine 2000 Reagent (Invitrogen, USA) following to manufacturer's instruction.

Real-time PCR

Total RNA for miRNA and mRNA analyses was isolated from tissues or cells using TRIzol reagent (Invitrogen; USA) according to the man-

ufacturer's information. The expression of miR-330-3p and MSI1 was determined with SYBR Green PCR master mix (Applied Biosystems, USA) using real-time PCR. The primers were used as following: MSI1 forward 5'-GCTCGAC-TCCAAAACAATTGACC-3' and reverse 5'-GGCTG-AGCTTTCTTACATTCCAC-3, and for GAPDH were forward 5'-TGTTCGACAGTCAGCCGC-3' and reverse 5'-GGTGTCTGAGCGATGTGGC-3'. GAPDH and U6 small nuclear RNA was performed as endogenous control for mRNA and miR-330-3p respectively.

Cell growth and colony-forming assay

Cell growth was determined using MTT assays according to the instructions. Cells were seeded in the 96-well plates and MTT reagent was put to the medium, and continue to culture for
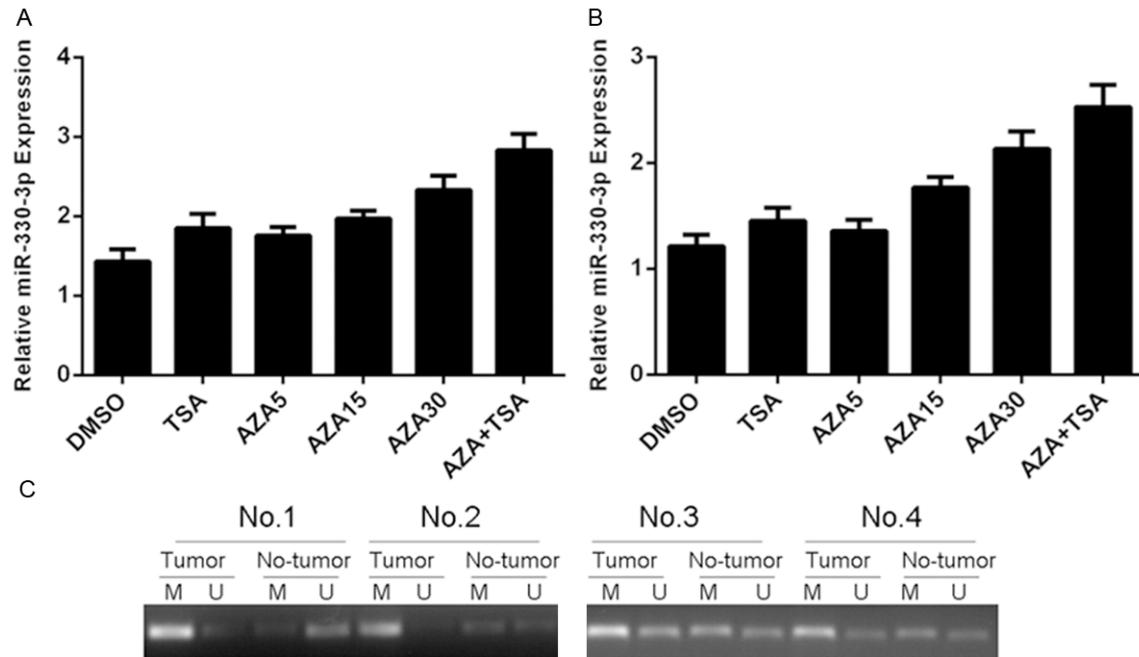
Figure 2. miR-330-3p expression was epigenetically regulated in gastric cancer. A: The expression of miR-330-3p in the HGC-27 cells after treated with TSA or AZA was detected by using qRT-PCR. B: The expression of miR-330-3p in the MGC-803 cells after treated with TSA or AZA was detected by using qRT-PCR. C: The methylation status of 4 pairs of GC tissues and adjacent no-tumor tissues was measured by MS-PCR.

more than 4 hours. The absorbance was measure on the microtiter plate reader (Molecular Devices, CA) at 490 nm. For colony formation analysis, cells were cultured in the 6-well plate for two weeks. Cell colony was fixed with paraformaldehyde (4%) and stained with crystal violet (1%) (Sigma-Aldrich, USA). Stained colony was counted under the microscope.

Cell migration assay

Wound-healing assay was performed to detect the cell migration. Cells were cultured in the six-well plates. The cell wound was scratched using the sterile plastic tip when cell was reached confluence. After wased with medium twice, cells were cultured for 48 hours and images were taken under microscope to measure the wound.

Luciferase assays

For luciferase assays, HGC-27 cell was cultured in the 24-well plate and transfected with reporter vector and miR-330-3p or scramble using Lipofectamine 2000 according to description. After 48 hours, the activitie of Renilla and firefly luciferase in the cell lysate was measured using

a dual-luciferase system (Promega). The ratio of Renilla/firefly luciferase activitie was calculated for normalization.

Western blotting

Total proteins were isolated from cell or tissue and protein concentrations were determined with the BCA protein assay kit (Pierce, USA). Proteins were separated using 12% SDS-PAGE gel and transferred to PVDF membranes (polyvinylidene difluoride; Bio-Rad). The membrane was blocked 5% non-fat milk and incubated with the primary antibody (E-cadherin, N-cadherin, vimentin and snail, MSI1 and GAPDH). The blot was incubated with horseradish peroxidase (HRP) for 1 hour and then was detected using ECL (enhanced chemiluminescence) system (Pierce Biotechnology, IL).

Sodium bisulfite modification and methylation-specific PCR (MS-PCR)

Total DNA from cell or tissues was isolated using Wizard DNA Purification Kit (QIAGEN, Valencia, CA) according to instruction. 10 ng DNA was used to PCR amplification and miR-330-3p CpG islands primers for unmethylated
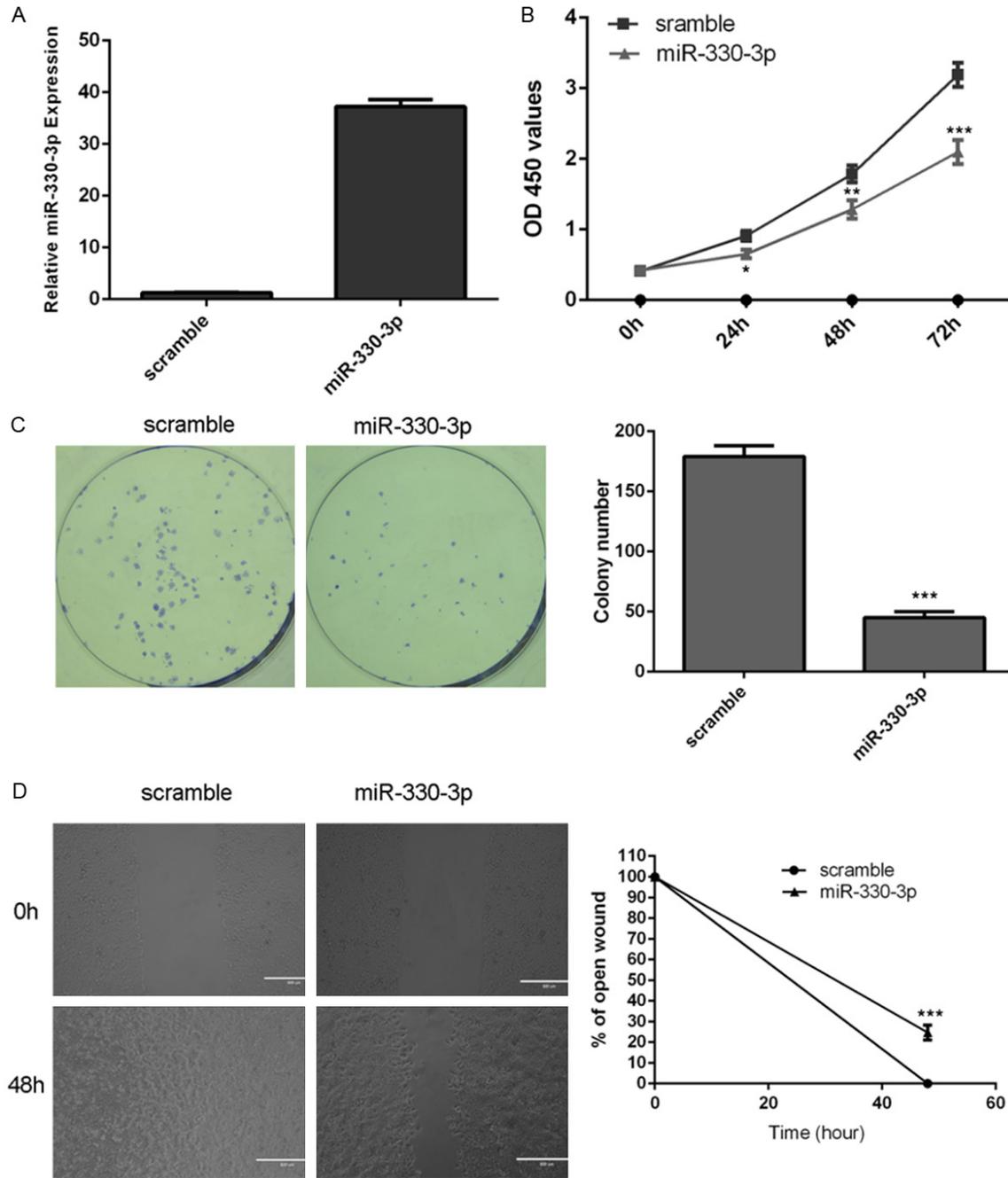
Figure 3. miR-330-3p inhibited gastric cancer cell proliferation, colony formation and migration. A: The expression of miR-330-3p was increased after treated with miR-330-3p mimic. B: Ectopic expression of miR-330-3p suppressed the HGC-27 cell proliferation using MMT assay. C: Overexpression of miR-330-3p inhibited the HGC-27 cell colony formation. D: miR-330-3p overexpression suppressed the HGC-27 cell migration. *P<0.05, **P<0.01 and ***P<0.001.

(forward: 5'-TGATTCGTTTTATTATCGGTC-3', reverse: 5'-AACACTAACGACATCGACG-3') and methylated (forward: 5'-TATGATTTGTTTTATTA-TTGGTT-30, reverse: 50-AACACTAACAACATCAACA ACC-3').

Statistical analysis

Data were shown as the mean ± SD (standard deviation). Statistical analysis was measured using SPSS 17. Student's t test was performed
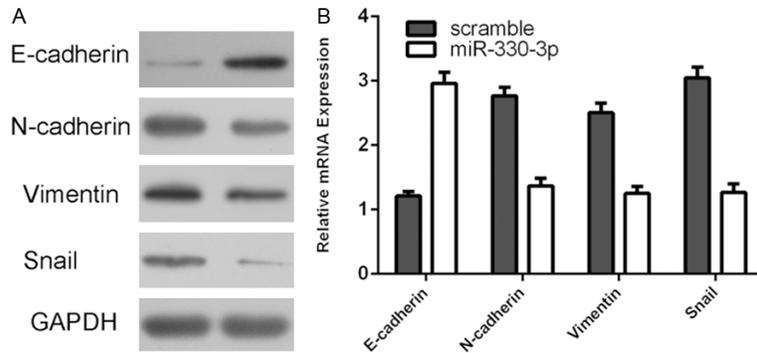
Figure 4. miR-330-3p repressed gastric cancer cell epithelial mesenchymal transition (EMT). A: The protein expression of E-cadherin, N-cadherin, vimentin and snail was measured by western blot. B: The mRNA expression of E-cadherin, N-cadherin, vimentin and snail was measured by qRT-PCR.
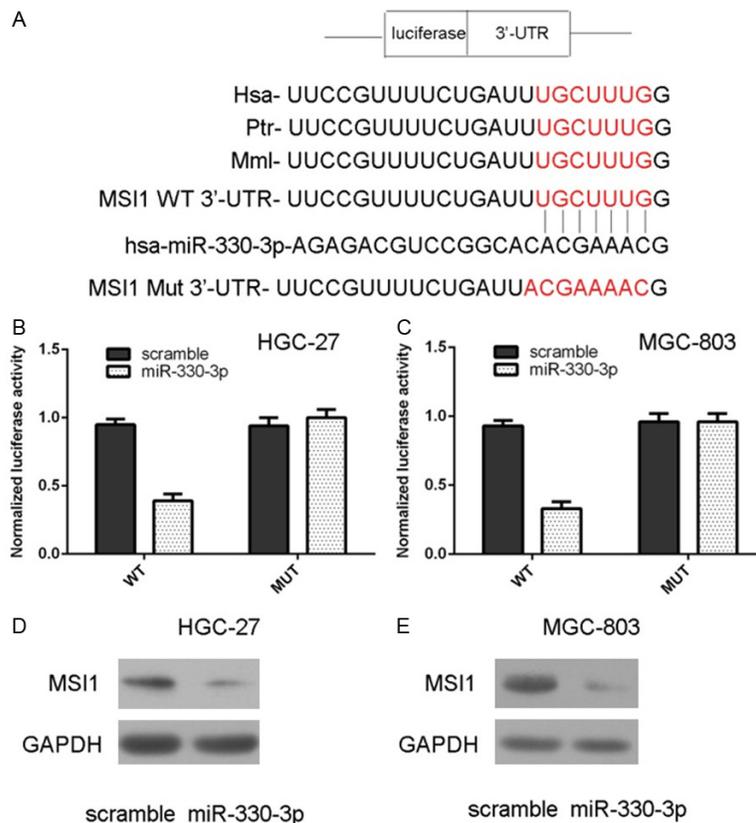
Figure 5. miR-330-3p targeted MSI1 in gastric cancer cell. A: There are 8 sequential bases between MSI1 gene 3' UTR and 5' of human miR-330-3p. B, C: Overexpression of miR-330-3p decreased the Luciferase activity of wild type MSI1 3' UTR construct, whereas the Luciferase activity was not decreased in the mutated type in both HGC-27 and MGC-803. D: Ectopic expression of miR-330-3p suppressed the protein expression of MSI1 in the HGC-27. E: The protein expression of MSI1 in the MGC-803 cell was determined by using western blot.

Result

miR-330-3p was downregulated in gastric cancer cell lines and tissues

The expression of miR-330-3p was determined in both GC tissues and cell lines. Of the 40 samples, miR-330-3p expression was downregulated in the 29 patients (29/40, 72.5%) compared to adjacent tissues (**Figure 1A**). The expression of miR-330-3p was downregulated in the GC tissues compared with adjacent no-tumor tissues (**Figure 1B**). We also demonstrated that miR-330-3p expression was downregulated in the GC cell lines (MGC-803, MNK-45, SGC-7901, HGC-27) compared with the normal gastric mucosa cell line (GES) (**Figure 1C**).

miR-330-3p expression was epigenetically regulated in gastric cancer

HGC-27 and MGC-803 cells were treated with the histone deacetylase inhibitor trichostatin A (TSA) and DNA methylation inhibitor 5-aza-CdR (AZA). miR-330-3p expression was increased in both HGC-27 and MGC-803 cells after treated with TSA or AZA (**Figure 2A** and **2B**). We also measured the methylation status of 4 pairs of GC tissues and adjacent no-tumor tissues by MS-PCR. The methylation level of GC tissues was higher than that of adjacent no-tumor tissues (**Figure 2C**).

miR-330-3p inhibited gastric cancer cell proliferation, colony formation and migration

The expression of miR-330-3p was increased after treated with miR-330-3p mimic (**Figure**

to measure differences and P<0.05 was considered significant.
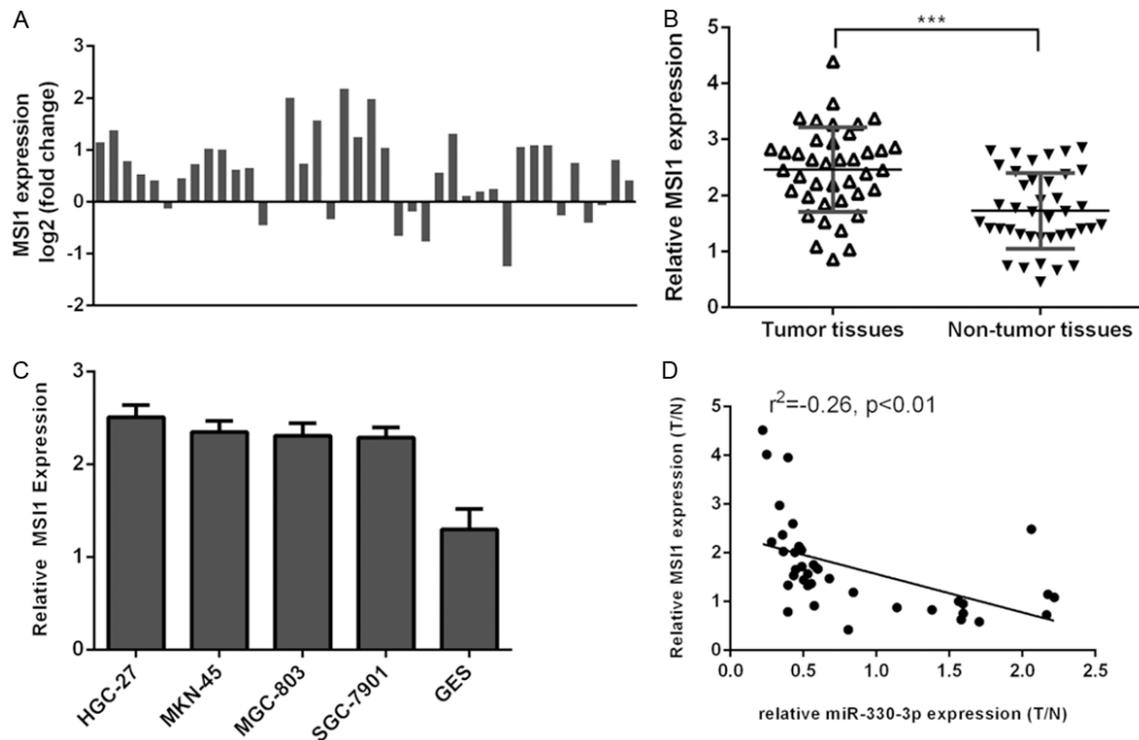
Figure 6. MSI1 expression was inversely correlated with miR-330-3p expression in gastric cancer. A: Of the 40 samples, MSI1 expression was upregulated in the 31 patients (31/40, 77.5%) compared to adjacent tissues. B: The expression of MSI1 was upregulated in the GC tissues compared with adjacent no-tumor tissues. C: The MSI1 expression in the GC cell lines (MGC-803, MNK-45, SGC-7901 and HGC-27) and the normal gastric mucosa cell line (GES) was determined by qRT-PCR. D: The expression levels of MSI1 were inversely correlated with the expression of miR-330-3p in the gastric cancer tissues. ***P<0.001.

3A). Ectopic expression of miR-330-3p suppressed the HGC-27 cell proliferation using MMT assay (**Figure 3B**). Overexpression of miR-330-3p inhibited the HGC-27 cell colony formation (**Figure 3C**). Moreover, miR-330-3p overexpression suppressed the HGC-27 cell migration (**Figure 3D**).

miR-330-3p repressed gastric cancer cell epithelial mesenchymal transition (EMT)

Overexpression of miR-330-3p promoted the protein expression of E-cadherin and repressed the protein expression of N-cadherin, vimentin and snail using western blot (**Figure 4A**). In line with this, ectopic expression of miR-330-3p enhanced the mRNA expression of E-cadherin and inhibited the mRNA expression of N-cadherin, vimentin and snail using qRT-PCR (**Figure 4B**).

miR-330-3p targeted MSI1 in gastric cancer cell

TargetScan online software was used to predict miR-330-3p genes and demonstrated that

MSI1 gene 3' UTR had 8 sequential bases with 5' of human miR-330-3p (**Figure 5A**). Overexpression of miR-330-3p decreased the luciferase activity of wild type MSI1 3' UTR construct, whereas the luciferase activity was not decreased in the mutated type in both HGC-27 (**Figure 5B**) and MGC-803 (**Figure 5C**). Ectopic expression of miR-330-3p suppressed the protein expression of MSI1 in both HGC-27 (**Figure 5D**) and MGC-803 (**Figure 5E**).

MSI1 expression was inversely correlated with miR-330-3p expression in gastric cancer

The expression of MSI1 was determined in GC tissues and cell lines. Of the 40 samples, MSI1 expression was upregulated in the 31 patients (31/40, 77.5%) compared to adjacent tissues (**Figure 6A**). The expression of MSI1 was upregulated in the GC tissues compared with adjacent no-tumor tissues (**Figure 6B**). We also demonstrated that MSI1 expression was upregulated in the GC cell lines (MGC-803, MNK-45, SGC-7901, HGC-27) compared with the normal gastric mucosa cell line (GES) (**Figure 6C**).

Moreover, the expression levels of MSI1 were inversely correlated with the expression of miR-330-3p in the gastric cancer tissues (**Figure 6D**).

Discussion

In this study, we showed that miR-330-3p expression was downregulated in gastric cancer cell lines and tissues. Ectopic expression of miR-330-3p suppressed the HGC-27 cell proliferation, colony formation and migration. Overexpression of miR-330-3p promoted the expression of E-cadherin and inhibited the expression of N-cadherin, vimentin and snail. These data suggested that miR-330-3p repressed gastric cancer cell epithelial mesenchymal transition (EMT). We identified MSI1 as a direct target gene of miR-330-3p in gastric cancer cell. In addition, MSI1 was upregulated in gastric cancer cell lines and tissues and the MSI1 expression was inversely correlated with miR-330-3p expression in gastric cancer tissues. Moreover, miR-330-3p expression was increased in GC cells after treated with histone deacetylase inhibitor trichostatin A (TSA) and DNA methylation inhibitor 5-aza-CdR (AZA). These indicated that downregulated expression of miR-330-3p was partly mediated by gene promoter region hypermethylation. To conclude, these results suggested that miR-330-3p acted as a tumor suppressor gene in GC.

Previous studies showed that miR-330-3p played an important role in the development of multiple tumors [33-38]. For example, Arora et al [38] found that the expression of miR-330-3p classified Brain metastasis (BM) + vs. BM-patients in nonsmall cell lung cancer patients. Lionetti et al [37] showed that deregulated expression of miR-330-3p in the serum existed in primary plasma cell leukemia and was correlated with clinical outcome. Liu et al [33] demonstrated that miR-330-3p expression was upregulated in NSCLC tissues and brain metastasis tissues. Overexpression of miR-330-3p promoted the NSCLC cell cycle distribution and proliferation through targeting EGR2. Meng et al [34] found that miR-330-3p expression was upregulated in esophageal squamous cell carcinoma (ESCC) tissues. miR-330-3p overexpression increased ESCC cell migration, invasion and proliferation through inhibiting PDCD4 expression. However, the role of miR-330-3p in the GC is still unknown. In our study, miR-330-3p expression was downregulated in GC cell lines and clinical GC tissues. Ectopic expression of miR-330-3p suppressed the GC cell proliferation, colony formation and migration.

MSI1 was identified as a candidate direct target of miR-330-3p by using the TargetScan. MSI1 original identified as one of neural stem cell (NSC) marker which is expressed in the nervous system [39, 40]. MSI1 is considered to be one marker of NSCs and neural progenitor cells in mammalian [41]. Moreover, higher expression of MSI1 was correlated with high grade glioma. Additionally, inhibition of MSI1 increased the radiation-induced colon cancer cells apoptosis and cancer regression. MSI1 is an RNA-binding protein (RBP) that plays an important role in the gene expression by binding to a sequence in the 3' UTR of target mRNAs leading to the translational inhibition [42-44]. Previous studies showed that MSI1 was overexpressed in the cervical cancer tissues and ectopic expression of MSI1 promoted the ectopic cell tumor formation, cell proliferation and increased the cervical cancer cells into the S phase [45]. MSI1 was expressed in a lot of tumors, including retinoblastoma, glioblastoma, and endometrial, colorectal, esophageal and bladder carcinoma [43, 46-51]. Morever, Wang et al [52] demonstrated that MSI1 expression was upregulated in GC tissues compare to normal gastric tissues. Kuang et al [53] also showed that MSI1 expression was upregulated in the intestinal metaplasia compared to the normal mucosa. It is reasonable to know that miR-330-3p target genes to inhibit cell proliferation and invasion. Among these putative target genes for miR-330-3p is an inhibitor of MSI1, which was predicted by the TargetScan. Our study proved that ectopic expression of miR-330-3p suppressed the protein expression of MSI1 in both HGC-27 and MGC-803 cell. Data from the Luciferase assays also suggest that MSI1 is one of the direct downstream target genes of miR-330-3p in the GC cell. In line with previous studies, we also found that the expression of MSI1 was upregulated in the GC tissues compared with adjacent no-tumor tissues. In addition, we found that the expression levels of MSI1 were inversely correlated with the expression of miR-330-3p in the gastric cancer tissues.

In conclusion, we demonstrated that miR-330-3p was downregulated in the human GC tissues. Ectopic expression of miR-330-3p suppressed the GC cell proliferation, colony formation and migration. MSI1 was identified as a direct target gene of miR-330-3p. These results suggested that miR-330-3p acted as a tumor suppressor gene in GC.

Disclosure of conflict of interest

None.

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