miR-29a-3p represses proliferation and metastasis of gastric cancer cells via attenuating HAS3 levels

FEIHU BAI[1*], MENGNA JIU[2*], YANJIE YOU[1*], YANING FENG[1], RUIJUAN XIN[1], XIAOGANG LIU[3], LIRONG MO[3] and YONGZHAN NIE[4]

[1]Department of Gastroenterology, Ningxia Hui Autonomous Region People's Hospital, Yinchuan, Ningxia Hui Autonomous Region 750021; [2]Department of Gastroenterology, Ankang Central Hospital, Ankang, Shanxi 725000; [3]Department of Basic Medicine, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region 750004; [4]Department of Gasteroenterology, Xijing Hospital of Digestive Diseases, State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xi'an, Shanxi 710000, P.R. China

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Abstract. MicroRNA-29a (miR-29a) has recently been in the spotlight as a tumor suppressor whose encoding gene is frequently suppressed in cancers. The aim of the present study was to investigate the biological functions and underlying molecular mechanism by which miR-29a-3p suppresses gastric cancer peritoneum metastasis. Cell proliferation, colony-forming, wound healing and Transwell migration assays were performed in the present study. MiR-29a-3p expression was markedly decreased in gastric cancer cell lines with stronger metastatic potential. Silencing miR-29a-3p expression promoted gastric cancer cell proliferation, colony-forming, migration and invasion. By contrast, overexpression of miR-29a-3p inhibited these biological phenotypes. In addition, it was revealed that miR-29a-3p functioned through down-regulating hyaluronan synthase 3 expression. Collectively, dysregulated miR-29a-3p expression in gastric cancer cells was associated with malignant properties primarily relevant to migration and metastasis. The results suggest that miR-29a-3p may be a potential therapeutic target for gastric cancer.

Introduction

As one of the most common malignancies and the second leading cause of cancer related deaths worldwide, gastric cancer is a heterogeneous disease with a variety of pathological entities and varied clinical behavior (1-3). There are about three hundred thousand newly diagnosed cases per year in China (4). Gastric cancer carries a poor prognosis that is largely attributable to early and frequent lymphatic, hematogenous metastasis and peritoneal dissemination, with a 5-year overall survival (OS) rate of less than 24% (5). Despite research endeavors and resources dedicated to elucidating the molecular mechanisms, and numerous genetic variants and genes with irregular expression discovered over the past decades, the precise molecular mechanisms of metastasis focused on gastric cancer is unclear and molecular markers for gastric cancer metastasis and tumor progression remain elusive. This ambiguity hampers the design of efficient and personalized chemotherapy and biotherapy strategies. Thus, finding metastasis-related genes and elucidating their function and clinical implication in gastric cancer are urgently demanded.

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs (~22 nucleotides in length) that negatively regulate the expression of multiple genes either by inducing translational silencing or by causing the degradation of messenger RNAs (mRNAs) of the targeted gene, via incomplete base-pairing to a complementary sequence in the 3'-untranslated region (3'-UTR) (6). In some settings, miRNAs also interact with amino acid coding regions of their mRNA targets (7). Increasing evidence shows that dysregulated miRNAs expression is involved in cancer progression and metastasis, and they might be the novel biomarkers or therapeutic targets in disease treatment (8-11).

During our efforts to discover new novel targets significantly associated with gastric cancer metastasis by integrative analysis of existing public data, we found that miRNA-29 (miR-29) family (miR-29a, miR-29b and miR-29c) can critically affect cancer progression by functioning as tumor suppressors (12). It has been found that the transcriptional levels of miRNA-29 are dramatically reduced in multiple cancer types (13-19), and are significantly correlated with patient survival (15-19). miRNA-29 acts as an integrator and an indispensable node of major signaling pathways, such as nuclear factor-κB signaling, and can mediates downstream signaling events that are involved in cancer cell motility and

Correspondence to: Dr Feihu Bai or Dr Yanjie You, Department of Gastroenterology, Ningxia Hui Autonomous Region People's Hospital, 301 Zhengyuan North Road, Yinchuan, Ningxia Hui Autonomous Region 750021, P.R. China
E-mail: baifeihu@sohu.com
E-mail: youyanjie@163.com

*Contributed equally

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invasion, cell cycle and apoptosis, epithelial mesenchymal transition (EMT) and chemoradiotherapy efficiency through multiple layers of mechanisms (14,16,20-26).

Recent evidence has revealed that the expression of miR-29 family members was significantly reduced in gastric cancer tissues compared with adjacent controls (14). miR-29b/c has been noted to form a cross-talk regulation with DNA methyltransferase 3A (DNMT3A), leading to the epigenetic silencing of CDH1 and subsequent metastasis phenotypes of gastric cancer (27). Also, it has been suggested that miR-29c activation may contribute to the chemotherapeutic-suppressed gastric cancer cell invasion (28). Nevertheless, to the best of our knowledge, the role of miR-29a in gastric cancer metastasis remains to be elucidated. Therefore, in this study we identified the expression profiles of miR-29a-3p, one transcripts of miR-29a (14), in gastric cancer cell lines with different metastatic potential. We then analyzed the biological functions of miR-29a-3p on gastric cancer cells and further verified its potential targets.

Materials and methods

Cell culture. Three pairs of high/low metastatic gastric cancer cell lines used in this study, denoted GC9811-p/GC9811, MKN28M/MKN-28NM, SGC7901M/SGC7901NM, were routinely cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 10 mmol/l glutamine, 100 units/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere containing 5% CO_2. The MKN28M and SGC7901 cell lines were amended to make them low metastatic and high metastatic as previously described (29,30).

RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA from cells in logarithmic phase was isolated using an E.Z.N.A.™ Total RNA Kit (Omega Bio-tek Inc, Norcross, GA, USA) according to the manufacturer's instructions. Quantified RNA (1 μg for miRNA 20 μl system and 500 ng for mRNA per 10 μl-systerm) was reverse transcribed using a PrimeScrip miRNA qPCR Starter kit for miRNA and a PrimeScript RT Master Mix reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 snRNA were used as an endogenous control. The primers for different PCR products are listed in Table I. qPCR was performed using a TaqMan Universal PCR Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) in an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 40 cycles of amplification reaction were allowed, each consisting of 95˚C 30 sec, 95˚C 5 sec, 60˚C 20 sec, 65˚C 5 sec for miRNA and 95˚C 30 sec, 95˚C 5 sec, 55˚C 30 sec, 72˚C 1 min, 72˚C 5 min, 65˚C 5 sec for mRNA. The formula $RQ=2^{-\Delta\Delta Cq}$ was used to calculate the relative expression levels.

Lentiviral infection and oligonucleotide transfection. The lentiviral GV369/GV280 vector was purchased from GeneChem Inc. (Shanghai, China), and the up and downstream flanking sequences of has-miR-29a-3p was insert into the vectors to overexpress its transcript. Lentiviral production was performed according to the manufacturer's instructions and puromycin (0.5 μg/ml) was used for selection. To RNA interference, pre-designed validated siRNA targeting miR-29a-3p and HAS3 were obtained from RiboBio (Guangzhou, China) and transfected into cells using a ribo FECT™ CP Transfection kit (RiboBio) following the manufacturer's protocol.

Table I. PCR primer sequences.

Primer	Sequence (5'-3')
miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
HAS3-forward	CAGCACTAAGGTGGACAGCA
HAS3-reverse	GGAGATGAAGGAAAGCACCA
GAPDH-forward	GCACCGTCAAGGCTGAGAAC
GAPDH-reverse	TGGTGAAGACGCCAGTGGA
U6-stem-loop	AGCGGGAAATCGTGCGTGACA
U6-reverse	GTGGACTFGGGAGAGGACTGG
Universal downstream	CGCCGCCCAGTGTTCAGA

PCR, polymerase chain reaction; HAS3, hyaluronan synthase 3.

Cell proliferation and colony formation assays. The cell proliferative and cologenic capacities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2'5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay and a colony formation assay respectively, according to standard methods described before (31,32).

Wound healing and transwell migration assays. The cell motility capacities were determined using a wound healing assay and a transwell chamber assay respectively, according to standard methods described before (33). All the experiments were performed in triplicate wells and repeated three times.

Western blot analysis. Total protein was extracted by RIPA buffer (Biyuntian, Beijing, China) mixed with PMSF (Roche Applied Science, Rotkreuz, Switzerland) in the rate 10:1. 30 μg of proteins were electrophoresed through 12% SDS polyacrylamide gels and transferred to a nitrocellulose filter membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h and then incubated with a rabbit PTEN antibody (catalog no. 9559; dilution 1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA) and a rabbit HAS3 antibody (catalog no. 15609-1-AP; dilution, 1;1,000; Proteintech Group Inc., Rosemont, IL, USA) in 4˚C overnight. A secondary HRP-conjugated goat anti-rabbit immunoglobulin G (IgG; catalog no. sc-2004; dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) incubated the membranes for 1 h and then the ECL western blotting analysis system (Bio-Rad installed with Quantity One; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect the signals. The blots were re-probed with β-actin monoclonal antibody (catalog no. bs-0061R; dilution, 1:1,000; Bioss, Beijing, China) to confirm equal loading of the different samples.

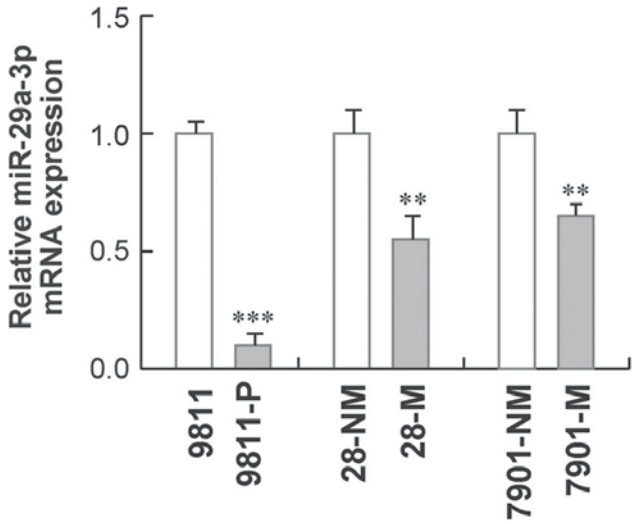

Figure 1. Expression analysis of miR-29a-3p levels in gastric cancer cells. Three pairs of gastric cancer cell lines (GC9811-p/GC9811, MKN28M/MKN-28NM and SGC7901M/SGC7901NM) were routinely cultured and total RNA was isolated from the cells was subjected to reverse transcription-quantitative polymerase chain reaction analysis using the specific primers. **P<0.01 and ***P<0.001 vs. the adjacent non-metastatic cell line. miR, microRNA.

Statistical analysis. All experiments were repeated at least three times in order to insure the repeatability. Measurement data were shown with the method of means ± standard deviation. A paired t-test was used for the comparison of two sample means. One-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post-test, was used to compare the means among multiple groups. A Chi-square test was used for the comparison of rate after the standardization. All statistical analyses were performed using the SPSS 15.0 statistical software package (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-29a-3p expression is down-regulated in high metastatic cell lines. Given that evidence that miR-29a-3p may act as a tumor suppressor, we first set out to examine the expression levels of miR-29a-3p in three pairs of gastric cell lines, each having different metastatic potentials. As shown in Fig. 1, miR-29a-3p expression levels appeared higher in cell lines with low metastatic potentials (GC9811, MKN-28NM and SGC7901-NM) than in their corresponding derived cell lines with high metastatic potentials (GC9811-p, MKN28-M and SGC7901-M). These data suggests that down-regulation of miR-29a-3p might associate with malignant properties mainly relevant to metastasis in gastric cancer cells.

miR-29a-3p regulates proliferation and migration of gastric cancer cells. Encouraged by the above data, we next performed gain- and loss-of-function analyses to clarify the biological functions of miR-29a-3p in terms of cancer cell proliferation and migration. Knockdown of miR-29a-3p and overexpression of miR-29a-3p were achieved in GC9811-p and GC9811 cells by lentiviral transduction, respectively, and confirmed by RT-qPCR analyses (Fig. 2A). As evidenced by MTT assays, we found that the proliferation rate of GC9811 cells with miR-29a-3p knockdown were significantly increased in comparison to their control cells. By contrast, GC9811-p cells with forced expression of miR-29a-3p grew slower than their control cells (Fig. 2B). Similarly, GC9811 cells with miR-29a-3p knockdown formed more colonies than their control, while GC9811-p cells with miR-29a-3p over-expression exhibited reduced colonies compared with their own control cells (Fig. 2C).

We further investigated the effect of miR-29a-3p on cell migration using a transwell migration assay. GC9811 cells with miR-29a-3p knockdown showed a significant increase in cell migration through the membrane of tranwell chambers than the control. As expected, GC9811-p cells with forced expression of miR-29a-3p achieved the opposite effects (Fig. 2D). The inhibitory effect of miR-29a-3p on cell migration was confirmed using a wound-healing assay. GC9811 cells with miR-29a-3p knockdown statistically significantly accelerated the closure of wound area, while GC9811-p cells with over-expression of miR-29a-3p delayed the wound closure, as compared with their controls respectively (Fig. 2E).

Bioinformatic target prediction of miR-29a-3p. In attempts to explore the molecular mechanisms through which miR-29a-3p might regulate gastric cancer progression, we searched for its putative target genes using three online prediction tools including Targetscan (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de/) and Miranda (http://www.microrna.org/microrna/home.do). The intersectional potential target genes were selected as shown in Fig. 3A. Among them, we screened out the hyaluronan synthase 3 (HAS3) gene which may be directly regulated by miR-29a. In this regard, the 3'UTR sequence UGGUGCUA upstream of both genes had perfect complimentarity with bases 2 through 8 counting from the 5'end of miR-29a-3p. We then examined whether miR-29a-3p expression was correlated with HAS3 by qRT-PCR analysis. By doing so, we observed that knockdown of miR-29a-3p in GC9811 cells obviously increased HAS3 mRNA levels, whereas overexpression of miR-29a-3p in GC9811-p cells decreased HAS3 expression (Fig. 3B). Altogether, these findings indicate that miR-29a-3p may negatively associate with HAS3 expression.

Knockdown of HAS3 suppresses gastric cancer cell proliferation and migration. To validate the biological functions of HAS3 in gastric cancer, we transfected a pre-designed and validated siRNA targeting HAS3 into GC9811-p cells, in which HAS3 was relatively highly expressed (Fig. 4A). We observed that GC9811-p cells with HAS3 knockdown showed a significant decrease in cell proliferation, as compared to those cells transfected with non-specific control siRNA (Fig. 4B). The effect of HAS3 on cell mobility was analyzed using a Transwell migration assay. The results showed that GC9811-p cells with HAS3 knockdown showed a significant decrease in cell migration compare to their control cells (Fig. 4C). Moreover, the wound healing assay revealed that silencing endogenous HAS3 in GC9811-p cells led to slower closure of the wound area than their control

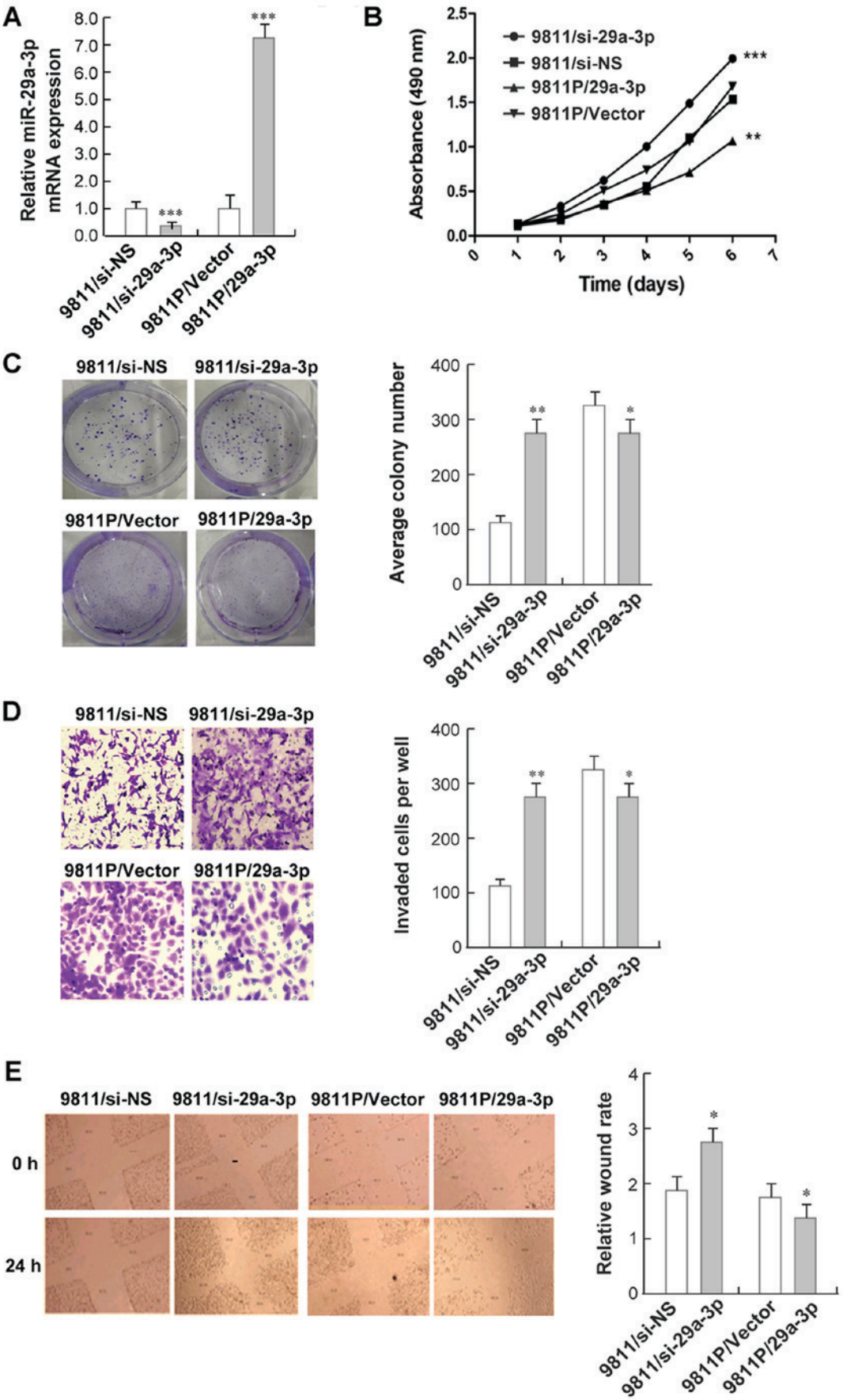

Figure 2. Dysregualted miR-29a-3p expression regulates proliferation and migration of gastric cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-29a-3p expression levels in GC9811 cells with miR-29a-3p knockdown and GC9811-p cells with miR-29a-3p overexpression. Subsequently, cell proliferation and mobility were assessed by (B) MTT assay, (C) colony formation assay, (D) Transwell migration assay and (E) would healing assay. Magnification, x100. *P<0.05, **P<0.01, ***P<0.001 vs. the adjacent control group. miR, microRNA; si, short interfering RNA; si-NS, non-specific control siRNA.

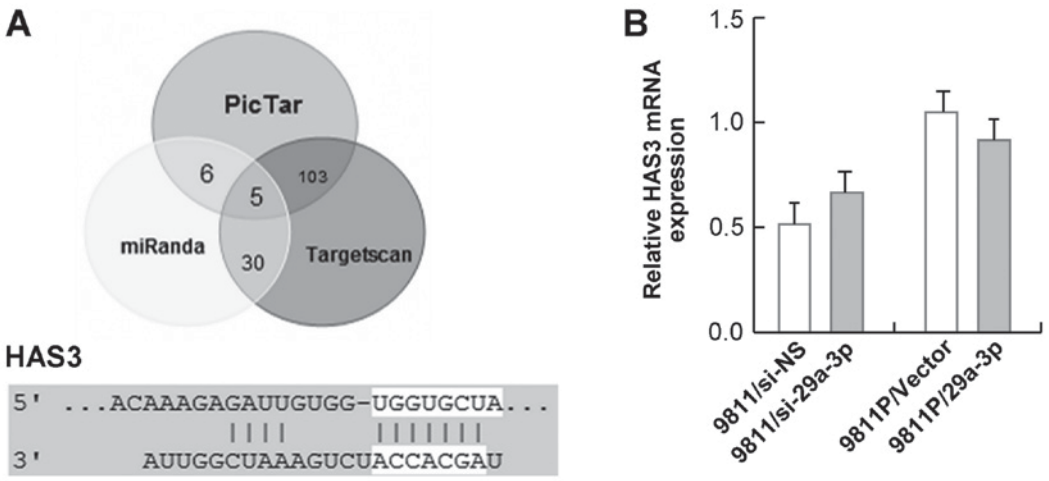

Figure 3. Bioinformatic target prediction of miR-29a-3p. (A) Bioinformatic target prediction was performed with three online tools and the intersectional potential target genes were shown. A putative miR-29a binding sequence is identified in the HAS3 3'UTR region. (B) Reverse transcription-quantitative polymerase chain reaction analysis of miR-29a-3p expression in GC9811 cells with miR-29a-3p knockdown and GC9811-p cell with miR-29a-3p overexpression. miR, microRNA; si, short interfering RNA; si-NS, non-specific control siRNA.

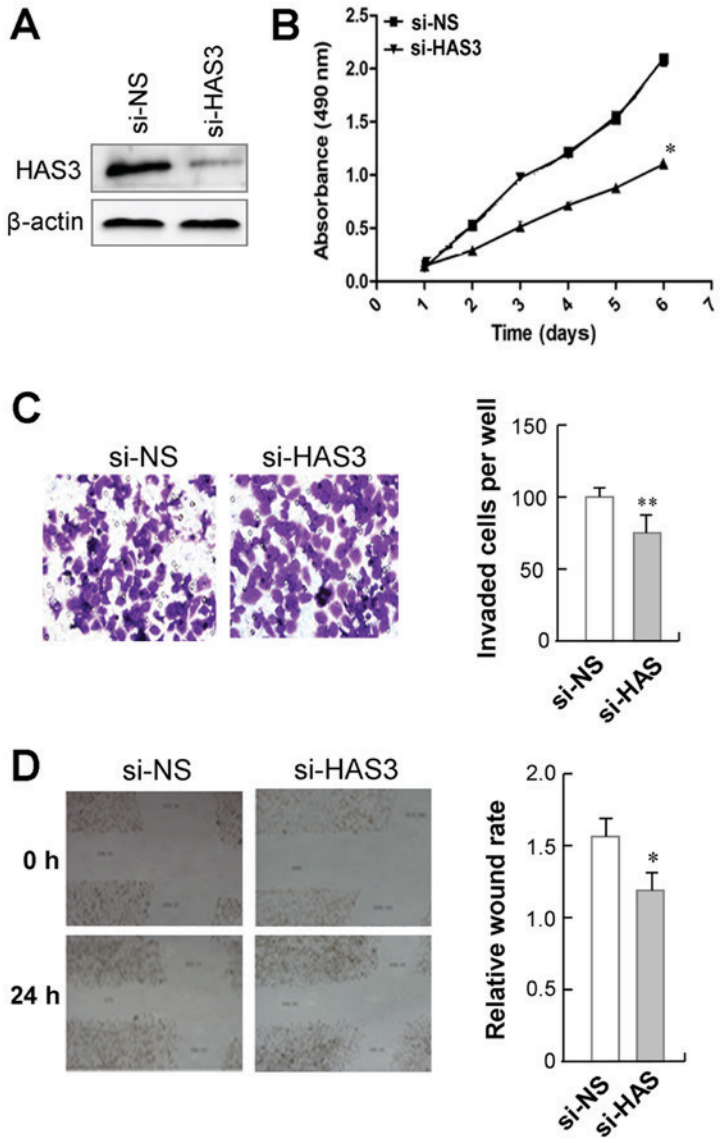

Figure 4. Knockdown of HAS3 suppresses GC9811 cell proliferation and migration. (A) Knockdown of HAS3 by siRNA in GC9811-p was confirmed by western blot analysis. Cell proliferation and mobility were assessed by (B) MTT assay, (C) Transwell migration assay and (D) would healing assay. Magnification, x100. *P<0.05, **P<0.01 vs. the adjacent control group. si, short interfering RNA; si-NS, non-specific control siRNA.

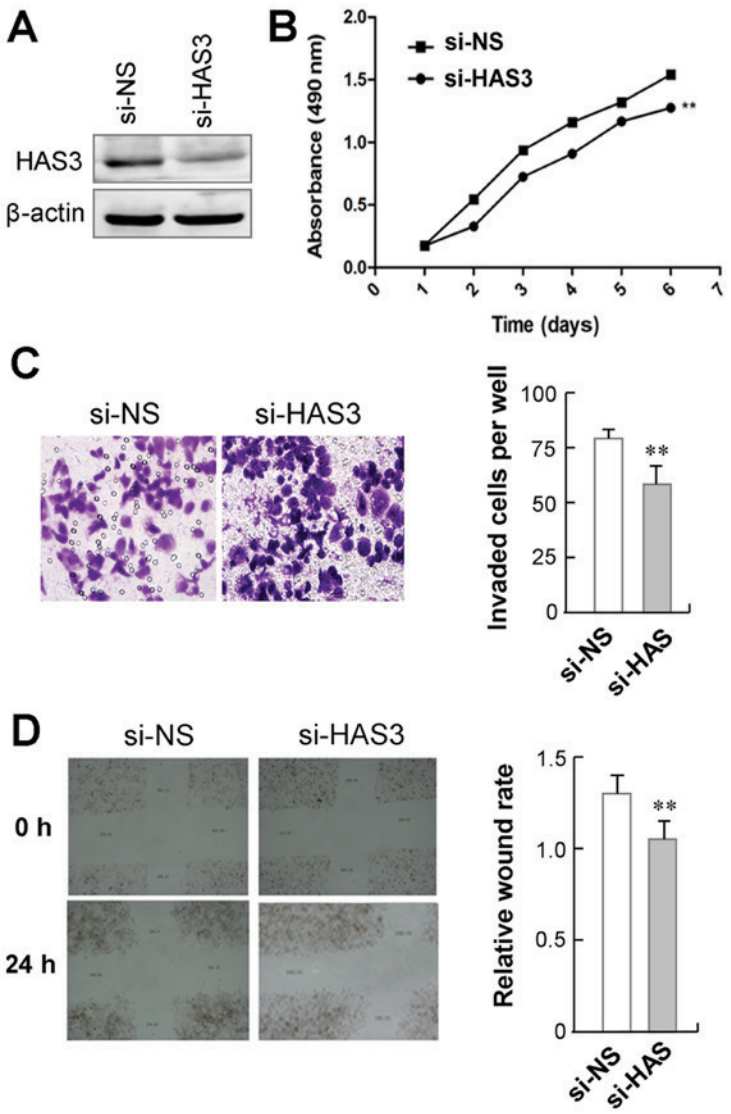

Figure 5. HAS3 knockdown resulted in a decrease in proliferation and metastatic potential of GC9811 cells with silencing of miR-29a-3p. (A) Knockdown of HAS3 by siRNA in GC9811 with silencing of miR-29a-3p was confirmed by western blot analysis. Cell proliferation and mobility were assessed by (B) MTT assay, (C) Transwell migration assay and (D) would healing assay. Magnification, x100. **P<0.01 vs. the adjacent control group. si, short interfering RNA; si-NS, non-specific control siRNA.

(Fig. 4D). Together, these findings clearly demonstrate that knockdown of CDK10 suppresses the proliferation and the migration GC9811-p cells.

HAS3 is crucial for miR-29a-3p-associated phenotypes in gastric cancer cells. Having observed that miR-29a-3p expression was negatively associated HAS3, we then carried out knockdown analysis was to confirm that HAS3 is required for miR-29a-3p-associated phenotypes in gastric cancer cells. GC9811 cells with miR-29a-3p knockdown were transfected with a HAS3-targeting siRNA (Fig. 5A), and the cell proliferation and metastasis abilities were measured. We found that silencing HAS3 expression led to a decreased proliferation rate in GC9811 cells with miR-29a-3p knockdown (Fig. 5B). Similarly, silencing HAS3 expression dramatically reduced the invasiveness and migration of GC9811 cells with miR-29a-3p knockdown, as determined by migration and would healing assays respectively (Fig. 5C and D). These data confirm that HAS3 is required for miR-29a-3p-associated phenotypes induced in gastric cancer cells.

Discussion

MiR-29a has been reported to have a close relation to many cancer types, such as malignant bile duct carcinoma, nasopharyngeal carcinoma and breast cancer. MiR-29 family members usually function as tumor suppressors by direct targeting oncogenic genes (13-19). In the present study, gastric cancer cell line GC9811 and its derived gastric line GC9811-p with high peritoneum metastasis potential were used in to analyze the biological functions of miR-29a-3p, one transcripts of miR-29a. We found that miR-29a-3p expression level was lower in GC9811-p cells than in GC9811 cells, indicating that miR-29a-3p may serve as a tumor-suppressive miRNA for gastric cancer metastasis. The observation was confirmed in other two pairs of high/low metastatic gastric cancer cell lines. Based on these observations, we further carried out the

gain- and loss-of-function analyses to clarify the biological functions of miR-29a-3p in terms of cancer cell proliferation and migration. The results indicate that miR-29a-3p can suppress gastric cancer cell growth and metastasis.

To understand the molecular mechanisms of miR-29a as a tumor suppressor in gastric cancer, we searched for putative miR-29a targets by online target gene prediction and HAS3 were selected out for subsequent research in this study. All family members of HSA proteins including HSA1, HSA2 and HSA3 have been closely linked to cancer progression and metastasis, in which HAS3 has the highest activity (34,35). It has been found that that the expression of HAS3 is conspicuous up-regulated in metastatic colon carcinoma cells and metastatic prostate cancer cells, in which the content of hyaluronic acid dramatically rise (36,37). Moreover, elevated HAS3 expression is found to be correlated with poor outcome for oral cancer and breast cancer (38,39). Overexpression of HAS3 could promote the proliferation of prostate cancer and melanoma cells, while knockdown of HAS3 inhibits the growth of colon cancer cells (40-42). Although its anti-cancer effect remains controversial (43), HAS3 has been postulated as a candidate tumor suppressor.

To validate the biological functions of HAS3 in gastric cancer, we silenced HAS3 by siRNA interference in GC9811-p cells with highly expressed HAS3 levels. As expected, knockdown of HAS3 could inhibit gastric cancer proliferation and metastasis. We proceeded to address the biological significance of the miR-29a-HAS3 connection in the regulation of gastric cancer cell penotypes. GC9811 cells with miR-29a-3p knockdown were transfected with a HAS3-targeting siRNA and then examined for cell growth, migration and invasion. Silencing of HAS3 significantly attenuated both proliferation and metastasis abilities of GC9811 cells with miR-29a-3p knockdown, suggesting that miR-29a-HAS3 axis is an important pathway in regulating growth and mobility anchorage-independent in gastric cancers and that miR-29a exerts its function, at least in part, through regulating HAS3 expression.

In summary, we demonstrate that miR-29a-3p can inhibit gastric cancer cells proliferation and metastasis by regulation HAS3 expression. These findings reveal a new mechanism by which, at least partially, miR-29a-3p may act as a candidate tumor suppressor. To the best of our knowledge, this is the first report that miR-29a-3p could regulate HAS3 expression, which broadens our understanding of miR-29a function. Our data suggest that miR-29a may have great potential in controlling tumorigenesis and metastasis, and further confirm that miR-29a is a promising target for gastric cancer prevention and therapy.

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Availability of data and materials

The dataset used and/or analyzed in the current study is available from the corresponding authors on reasonable request.

Authors' contributions

MJ, RX, XL YF and LM performed the experiments, analyzed the data and participated in the experiment design and manuscript writing. YN contributed to the analysis and interpretation of data. FB and YY conceived the study, designed the experiments and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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