miR-181a-5p promotes the progression of gastric cancer via RASSF6-mediated MAPK signalling activation

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Abstract

We previously discovered that Ras association domain family member 6 (RASSF6) was downregulated and predicted poor prognosis in GC patients. However, the mechanisms of the down regulation of RASSF6 in GC remained unclear. Increasing evidence indicates that dysregulation of microRNAs promotes the progression of cancer through the repression of tumour suppressors. Here, we identified miR-181a-5p as a novel regulator of RASSF6 in GC. Functionally, ectopic expression or silencing of miR-181a-5p, respectively, promoted or inhibited GC cell proliferation, colony formation and cell cycle transition, as well as enhanced or prevented the invasion, metastasis of GC cells as well as enhanced or prevented the invasion, metastasis of GC cells and epithelial to mesenchymal transition of GC cells in vitro and in vivo. Molecularly, miR-181a-5p functioned as an onco-miRNA by activating the RASSF6-mediated MAPK pathway. Overexpression or silencing of RASSF6 could partially reverse the effects of the overexpression or repression of miR-181a-5p on GC progress caused by activation of the MAPK pathway in vitro and in vivo. Clinically, high miR-181a-5p expression predicted poor survival in GC patients, especially combined with low RASSF6 expression. Collectively, we identified miR-181a-5p as an onco-miRNA, which acts by directly repressing RASSF6 in GC.

Introduction

Gastric cancer (GC) is the second most common cancer-related mortality throughout the world [1] due to its rapid progression to advanced stages and highly metastatic properties [2]. Currently, no efficient biomarker that can predict the recurrence and metastasis of GC can be widely used, except for traditional TNM staging in clinics [3]. Hence, the genetic alterations and epigenetic changes (and their mechanisms) involved in GC should be explored more intensively to discover prognostic biomarkers for GC.

We previously reported that Ras association domain family member 6 (RASSF6) was downregulated in GC by using a loss of heterozygosity (LOH) analysis and cDNA microarrays [4]. We also discovered that decreased expression of RASSF6 was a marker of poor prognosis in GC [5]. Others also showed that RASSF6 acts as a tumour suppressor and exhibits high DNA methylation and downregulation in other cancers [6,7]. Therefore, it is important to study the mechanisms involved in the downregulation of RASSF6 in GC. Recently, microRNAs, a group of small non-coding RNAs that can suppress gene expression by interacting with the 3’ untranslated regions (UTRs) of target mRNAs, have been widely studied in...
cancer [8]. In GC, many microRNAs have been discovered and have been found to regulate several target genes and act as tumour promoting or suppressing markers [9]. Hence, microRNAs that are involved in the progression of GC are of particular interest as potential prognostic biomarkers in GC.

In this study, miR-181a-5p was identified as a regulator of RASSF6 in GC. High expression of miR-181a-5p in tumour tissues of GC was positively correlated with cancer proliferation and metastasis in GC patients. Mechanistically, miR-181a-5p promotes GC cells proliferation, invasion, metastasis and epithelial-to-mesenchymal transition (EMT) in vitro and in vivo through activation of the RASSF6/MAPK signalling pathway.

Materials and methods

Details are described in the Supplementary materials and methods.

Results

Identification of miR-181a-5p as a negative regulator of RASSF6 in human gastric cancer

To explore the potential mechanism of the down regulation of RASSF6 in GC, we first evaluated the genetic or epigenetic dysregulation of RASSF6 in GC by using public data from The Cancer Genome Atlas (TCGA). However, we only found few data regarding the dysregulation of RASSF6 at the genetic and epigenetic levels (Fig. S1 and S2, Table S1), suggesting that genetic mutations and epigenetic modifications were not the main cause of the RASSF6 decrease in GC. An increasing number of studies has revealed that miRNAs play an important role in GC progression by directly interfering with the expression of their targets, which suggests that RASSF6 might be regulated by miRNAs in GC [10]. Then, we used the TargetScan 7.0, StarBase v2.0 and microRNA.org software to predict the potential miRNAs that could directly target RASSF6 3’-UTR. Only miRNAs that could bind to the same position in the sequence of the RASSF6’s 3’-UTR, as identified from the 3 programs used, were selected for further investigation. We discovered that 4 miRNAs had the same binding sites in the 3’-UTR of RASSF6 (Table S10).

To evaluate the roles of these 4 miRNAs on RASSF6 expression, luciferase reporter vectors containing the wild-type RASSF6 3’-UTR and respective mimics of those 4 miRNAs were co-transfected into 293T cells. Interestingly, the luciferase activity significantly decreased only in the group that was transfected with the miR-181a-5p mimics compared with the control group (Fig. 1A). These data suggested that miR-181a-5p might be a regulator of RASSF6 in GC cells. To investigate the roles of miR-181a-5p on the
progression of GC, we randomly selected 30 GC patients with and without distant metastasis and evaluated the expression levels of miR-181a-5p and RASSF6 in tumour tissues. We found that miR-181a-5p was more highly expressed in tumours with distant metastasis than in those without metastasis (Fig. 1B). Conversely, RASSF6 expression was lower in tumours with distant metastasis than in those without metastasis (Fig. 1C). A Spearman correlation analysis showed that miR-181a-5p expression was negatively associated with the RASSF6 mRNA level in these patients’ tumour tissues (Fig. 1D). To further investigate whether miR-181a-5p could play a significant role in GC by regulating RASSF6, one normal gastric mucosa epithelial cell line (GES-1) and 6 GC cell lines were used to evaluate the expression level of miR-181a-5p. Like the normal gastric mucosa epithelial cell line (GES-1), the MKN-28 cell line, which is derived from well-differentiated GC tumours, also presented with low miR-181a-5p expression, while the HGC-27 cell line, which comes from poorly differentiated GC tumours, showed the highest miR-181a-5p expression (Fig. 1E). In addition, we evaluated the expression profile of RASSF6 in GES-1 cells as well as in the 6 GC cell lines, both at the protein and mRNA levels, using western blot and qPCR. Both the western blot and qPCR assays showed that RASSF6 was highly expressed at the protein and mRNA levels in the GES-1 and MKN-28 cell lines, while it was expressed at the lowest level in the HGC-27 cell line (Fig. S3A and S3B). We chose the MKN-28 and HGC-27 cell lines for our research. Interestingly, both the miR-181a-5p mimics and inhibitors had a dose dependent effect on the luciferase activity when investigating the effect of miRNAs on the RASSF6 3’UTR (Fig. 1F and G), indicating that miR-181a-5p might directly regulate the RASSF6 3’UTR in those cell lines. Additionally, overexpression or knockdown of miR-181a-5p downregulated or upregulated, respectively, RASSF6 at both the mRNA and protein levels (Fig. 1H and I). Taken together, these data indicate that miR-181a-5p negatively regulates RASSF6 in human GC and might play an important role in the progression of this disease.

miR-181a-5p promotes GC cell proliferation, wound healing, and invasion and induces EMT in vitro

To explore the potential functions of miR-181a-5p in the tumourigenesis of GC, miRNA mimics or anti-mimics were transfected into MKN-28 or HGC-27 cells, respectively. The effects of exogenous miR-181a-5p mimics and anti-mimics were confirmed by qPCR and RT-PCR (Fig. S4A and S4B). The 5-day in vitro cell growth assay, using a CCK8 kit, showed that overexpression or knockdown of miR-181a-5p significantly promoted or inhibited MKN-28 or HGC-27 cell proliferation, respectively, compared with their negative controls (Fig. 2A and B). A colony formation assay further confirmed the role of miR-181a-5p in promoting proliferation of GC cells (Fig. 2C and D). Furthermore, we also demonstrated that overexpression or knockdown of miR-181a-5p generated cell cycle changes with a smaller proportion of cells in the G0/G1 phase and a larger proportion of cells in the S-phase when miR-181a-5p was overexpressed; the opposite results were
observed when miR-181a-5p was silenced (Fig. 2E and F). Cyclin D1 and c-myc play significant roles in cell cycle changes during the transition from the G0/G1 phase to the S phase. We also demonstrated that overexpression or knockdown of miR-181a-5p resulted in upregulation or downregulation of CyclinD1 and c-myc proteins, respectively (Fig. 2G). These results suggested that miR-181a-5p could promote GC cells proliferation by inducing the transition to the S phase of the cell cycle.

Subsequently, wound healing and transwell assays showed that overexpression or knockdown of miR-181a-5p could significantly strengthen or weaken, respectively, the migration and invasion abilities of GC cells (Fig. 3A–D). Collectively, these results highlighted that miR-181a-5p could boost GC cell migration and invasion ability in vitro. MMP2 and MMP9, markers of cell migration and invasion, play an important role during miR-181a-mediated cancer progression [11,12]. Our study showed that overexpression or knockdown of miR-181a-5p, respectively, increased or decreased the MMP2 and MMP9 protein levels in GC cells (Fig. 3E). Epithelial-to-mesenchymal transition (EMT) is a crucial step during cancer cell metastasis [13]. We found that upregulation of miR-181a-5p in MKN-28 cells resulted in low expression of an epithelial marker (E-cadherin) and high expression of a mesenchymal marker (N-cadherin), both at the mRNA and protein levels (Fig. 3F and H), while downregulation of miR-181a-5p in HGC-27 cells led to the opposite effects (Fig. 3G and I). These discoveries indicated that miR-181a-5p could induce EMT in GC cells and promote invasion and metastasis of GC cells in vitro.

miR-181a-5p accelerates GC cells growth in vivo

To confirm the contribution of miR-181a-5p in tumourigenesis, we further performed subcutaneous tumour xenograft assays using miR-181a-5p-overexpressing or miR-181a-5p-silenced cells (or their negative control cells) in BALB/c nude mice. Overexpression or silencing of miR-181a-5p in GC cells correspondingly generated larger or smaller xenografts, which were measured by the tumour weights and volumes in nude mice compared to their control (Fig. 4A and B). Notably, the average levels of miR-181a-5p in the subcutaneous tumour xenografts generated by the injection of miR-181a-5p-overexpressing or -silenced cells were significantly higher or lower than their controls, respectively (Fig. 5A). In addition, immunohistochemical (IHC) staining also verified that miR-181a-5p overexpression or knockdown in the xenografts was associated with obviously stronger or weaker Ki67 staining, respectively (Fig. 4C and D). These results suggested that miR-181a-5p played a significant role in promoting the tumour formation and growth in vivo.

miR-181a-5p facilitates invasion, metastasis and EMT of GC cells in vivo

Using in vitro cell function assays, we investigated whether miR-181a-5p could affect the migration and invasion of GC cells and whether it could induce EMT in vivo. A peritoneal dissemination assay showed a significant increase or decrease in the number

Fig. 3. miR-181a-5p promoted invasion, EMT and induced the variations of proteins associated with these processes in vitro. (A-B) Wound healing assays were performed to investigate the changes of GC cells’ migration ability upon overexpression or knockdown of miR-181a-5p compared with their negative controls, respectively. (C-D) Transwell invasion assays were used to estimate the effects of miR-181a-5p up- or down-regulation on the GC cells’ migration and invasion abilities. The percent of wound closure or number of cells passed through the membrane was counted and compared in the diagrams. (E) Western blot analyses revealed that overexpression or silencing of miR-181a-5p significantly increased or decreased MMP2 and MMP9 expression, respectively. (F-G) Changes in E-cadherin and N-cadherin mRNA in miR-181a-5p-overexpressing MKN-28 cells, miR-181a-5p-silenced HGC-27 cells and their controls were analysed by qPCR assay. (H-I) Levels of E-cadherin and N-cadherin proteins upon up- or down-regulation of miR-181a-5p were determined by western blot in GC cells. (*p < 0.05; **p < 0.01; ***p < 0.001).
metastatic nodules in the peritoneal cavity upon injection of an equal number of GC cells overexpressing or with knocked-down miR-181a-5p compared with their controls (Fig. 5A and B). We obtained metastatic colonies from the peritoneal cavity and evaluated the expression of miR-181a-5p in fresh metastatic colonies of each group. Expression of miR-181a-5p was higher in the peritoneal dissemination metastatic nodules of mouse-injected cells transfected with miR-181a-5p overexpressing vectors, while it was lower in the peritoneal dissemination metastatic nodules of mouse-injected cells transfected with anti-miR-181a-5p mimics compared with controls, respectively (Fig. 5B). We used IHC staining to evaluate MMP2 and MMP9 expression in paraffin tissue blocks of metastatic nodules and found that tissues from miR-181a-5p-overexpressing or -silenced GC cells showed stronger or weaker MMP2 and MMP9 staining, respectively, compared to controls (Fig. 5C and D). In addition, IHC staining showed that upregulation of miR-181a-5p was positively associated with weaker E-cadherin and stronger N-cadherin staining compared with the control (Fig. 5E). Conversely, downregulation of miR-181a-5p was positively correlated with stronger E-cadherin and weaker N-cadherin staining (Fig. 5F). In summary, these data suggested that miR-181a-5p could promote GC cell invasion, metastasis and EMT in vivo.

miR-181a-5p activates the MAPK signalling pathway by directly inhibiting the expression of RASSF6

As shown in Fig. 6a, there is a RASSF6 3’UTR-binding site in the miR-181a-5p sequence (Fig. 6A). Double-luciferase reporter assays showed that the luciferase activity of the plasmid containing the wild-type RASSF6 3’UTR was significantly decreased in the presence of wild-type miR-181a-5p but not of mutant miR-181a-5p. Conversely, the luciferase activity of mutant RASSF6 3’UTR was obviously reduced in the presence of mutant miR-181a-5p but not in the presence of wild-type miR-181a-5p (Fig. 6B). Additionally, downregulation of miR-181a-5p resulted in increased luciferase activity of the plasmid containing the wild-type RASSF6 3’UTR.

Fig. 4. miR-181a-5p accelerated GC cells growth in vivo. (A-B) Overexpression or silencing of miR-181a-5p, respectively, generated larger or smaller subcutaneous tumour xenograft than control groups in nude mice. Tumours were compared in weight and volume, and the volume was calculated with the formula, volume = length x width x length x 1/2 (n = 5, *p < 0.05, **p < 0.01). (C-D) Representative IHC images of Ki-67 staining in miR-181a-5p-overexpressing MKN-28 cells, miR-181a-5p-silenced HGC-27 cells and their negative controls. Original magnification, ×200; bar, 100 μm.
miR-181a-5p facilitated peritoneal dissemination of metastasis and induced EMT of peritoneal dissemination nodules in nude mice. (A) Representative photographs of peritoneal dissemination (A1: normal control; A2: tumour colonies in the peritoneal cavity of nude mice). The arrowheads point to the tumour nodules in the peritoneal cavity. (B) The number of peritoneal dissemination metastatic nodules in mice was counted with a dissecting microscope. *p < 0.05, **p < 0.01. (C) IHC staining analyses showed the staining of MMP2 and MMP9 in peritoneal dissemination tumour nodules upon miR-181a-5p upregulation or downregulation and the controls. Overexpression or inhibition of miR-181a-5p significantly strengthened or weakened MMP2 and MMP9 staining, respectively. (D) Representative E-cadherin and N-cadherin IHC staining images of peritoneal dissemination tumour colonies, generated by injecting miR-181a-5p overexpressing MKN-28 cells or miR-181a-5p silenced HGC-27 cells, as well as their control groups. Original magnification, ×200; bar, 100 μm.
Interestingly, the luciferase activity of the MAPK pathway reporter array to investigate the molecular mechanisms through which miR-181a-5p regulates GC cell proliferation and metastasis. Interestingly, the luciferase activity of the MAPK pathway reporter gene was significantly increased or decreased by overexpressing miR-181a-5p or knocking down its expression (Fig. 6D and E). Moreover, a bioinformatics prediction using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases also indicated that miR-181a-5p might be involved in the MAPK pathway in GC (Fig. S6 and S7; Table S11 and S12). To further investigate whether miR-181a-5p actually activates the MAPK signalling pathway in GC cells via RASSF6, we constructed vectors that overexpressed or knocked down RASSF6 in GC cells, the efficiencies of which were examined by qPCR and western blot (Fig. S8A and S8B). The western blot assay showed that p-P38 and p-ERK were high or low expressed upon ectopic expression or knockdown of miR-181a-5p, and this effect could be reverted by re-introduction or inhibition of RASSF6, respectively (Fig. 6F and G).

Furthermore, a specific inhibitor (SB203580) of p-P38 MAPK could significantly reverse the p-P38 and p-ERK levels induced by overexpression of miR-181a-5p (Fig. 6H). These results supported the idea that miR-181a-5p actually activated the MAPK signalling pathway by directly targeting RASSF6 in GC cells and therefore had an effect on the proliferation and metastasis of GC.

**Ectopic expression of miR-181a-5p promotes GC cell proliferation and metastasis by targeting RASSF6 in vitro and in vivo**

To validate the effects of miR-181a-5p on RASSF6 in GC, we further applied the described functional assays in vitro and in vivo. As shown in Fig. 7, the effects of miR-181a-5p up- or down-regulation on GC cells in promoting or inhibiting colony formation and invasion were significantly reversed by co-transfection of the Lv-RASSF6 or RASSF6 siRNA vectors in vitro (Fig. 7A–D, Fig. S9A-S9D). In vivo peritoneal metastasis experiments were performed by injecting miR-181a-5p-overexpressing or -silenced GC cells co-transfected with vectors overexpressing or knocking-down RASSF6 vectors into nude mice, respectively. We found that overexpression or knockdown of RASSF6 could partially counteract the effects of up- or down-regulation of miR-181a-5p on the peritoneal metastatic ability of nude mice, respectively, compared with their controls, (Fig. 7E and F). Collectively, these in vitro and in vivo results demonstrated that ectopic expression of miR-181a-5p could facilitate GC cell proliferation and metastasis by directly suppressing RASSF6.

**Upregulation of miR-181a-5p was negatively associated with RASSF6 in human GC tissues and indicates poor survival in GC patients**

Clinically, we used the publicly available TCGA dataset and data from the Shanghai General Hospital for analyses. Both the data from TCGA and the Shanghai General Hospital showed that the level of miR-181a-5p was significantly higher in tumour tissues than in normal tissues (Fig. 8A and B, Table S13). Analyses using data from the Shanghai General Hospital further demonstrated that high miR-181a-5p expression was negatively correlated with RASSF6 protein levels in 260 GC tumours (Fig. S10A and S10B; Table S14). There were 139 (53.5%) GC patients who showed high miR-181a-5p levels, and 121 (87%) of these patients presented negative or weak RASSF6 IHC staining in tumour tissues (Table S14). However, of the 121 (47.5%) cases with low miR-181a-5p expression, only 38 (31.4%) patients' tumour tissues presented negative/weak RASSF6 staining (Table S14). This evidence further supports that miR-181a-5p negatively correlated with RASSF6 in GC.

Subsequently, we analysed the correlation among miR-181a-5p and clinical characteristics using the data from the Shanghai General Hospital. High miR-181a-5p expression significantly affected the tumour size, TNM stage, UICC stage, vessel and nerve invasion (p < 0.05 for all, Table S14). There was no significance regarding the age, gender or tumour location (p > 0.05 for all, Table S14). These findings suggested that upregulation of miR-181a-5p was involved in the tumourigenesis and progression of GC, which may affect the prognosis of GC.

Next, a Kaplan–Meier analysis with a log rank test on tumour recurrence free survival (RFS) using two data points showed that high miR-181a-5p predicted worse RFS in GC patients (Fig. 8C and D). The overall survival (OS) analyses of the data from the non-recurrence and recurrence groups from the Shanghai General Hospital revealed that high miR-181a-5p expression alone predicted poorer OS in the recurrence group, but not in the non-recurrence patients (Fig. 8E and F). Univariate and multivariate survival analyses further indicated that miR-181a-5p was a high risk factor for poor prognosis of GC patients, especially in tumour recurrence (Table S15). Furthermore, patients with high miR-181a-5p expression and negative/weak RASSF6 staining had lower RFS and OS rates than those with lower miR-181a-5p expression (Fig. S11A and S11B). More interestingly, both the non-recurrence and recurrence group patients presented the worst OS rates if their tumours had a high miR-181a-5p level and negative/weak RASSF6 staining (Fig. S12A and S12B). Together, high miR-181a-5p combined with low RASSF6 expression was an indicator of poor survival in GC patients.

**Discussion**

We previously found that RASSF6 was down regulated in GC [4]. Low RASSF6 predicted poor prognosis for GC patients [5]. The present study further determined miR-181a-5p to be a new modulator of RASSF6 in GC. High expression of miR-181a-5p promoted the proliferation, invasion and peritoneal dissemination of metastasis of GC cells via RASSF6-mediated activation of the MAPK pathway in vitro and in vivo. More importantly, high miR-181a-5p together with low RASSF6 expression predicted poor prognosis in GC patients.

Tumour recurrence and metastasis are the main reasons for the poor prognosis of GC patients after radical surgery [18]. Discovering and using biomarkers that can efficiently evaluate and identify the risks of tumour recurrence and metastasis in GC is important. Previously, we identified RASS6 as a tumour suppressor in GC. Others have reported that RASS6 is a tumour suppressor with a high frequency of DNA methylation in childhood leukaemia and neuroblastoma [7]. Meanwhile, our analyses of genetic or epigenetic dysregulation of RASS6 using TCGA data suggested that genetic mutations and epigenetic modifications were not reasons for the reduced expression of RASS6 in GC. Investigations of miRNA expression provided other insights into the mechanisms of GC carcinogenesis and development, contributing to the discovery of new treatment targets. Therefore, we hypothesized that endogenous miRNAs might regulate RASS6 expression in GC.

Indeed, our subsequent bioinformatics analyses and luciferase reporter assays demonstrated that miR-181a-5p might function as a novel repressor of RASS6 in GC. Meanwhile, these findings were challenged by Li's report indicating that miR-181a-5p could inhibit the migration and angiogenesis of cancer cells via down-regulation of MMP14 [19]. Recently, it was found that high levels of miR-181a-5p could promote the progression of ovarian cancer via Smad7-mediated activation of the TGFβ signalling pathway and...
miR-181a-5p activated the MAPK signalling pathway by directly targeting the 3’ UTR of RASSF6 in GC cells. (A) The corresponding sequences of predicted binding sites in the wild type/mutant 3’UTR of RASSF6 and miR-181a-5p. All of the luciferase reporter vectors were constructed as described in Supplementary Materials and Methods. (B-C) Overexpression or silencing of miR-181a-5p decreased or increased the luciferase activity of the vector containing the wild-type 3’UTR of RASSF6, but not that of the vector with the mutated 3’UTR, while the mutant miR-181a-5p inhibited the luciferase activity. Empty mimics or anti-mimics were used as negative controls (**p < 0.01; ***p < 0.001). (D-E) Multi-pathway reporter arrays were used to explore, in GC cells, the possible signalling pathways miR-181a-5p was involved in. (F-G) Western blot analyses were performed to compare the differences among the representative makers of the MAPK pathway in miR-181a-5p overexpressing MKN-28 cells. (H) Transfection of RASSF6 siRNA plasmids partially reduced the negative effect of miR-181a-5p downregulation on p-P38 and p-ERK proteins. (H) Effect of a specific inhibitor of p-38 MAPK (SB203580) on the results of miR-181a-5p overexpression in the MAPK signalling.
Fig. 7. miR-181a-5p promoted the colony formation, invasion and metastasis by directly downregulation of RASSF6 in vitro and in vivo. The empty control and Lv-RASSF6 plasmids were transfected into miR-181a-5p-overexpressing or control MKN-28 cells, respectively. Scramble and RASSF6 siRNA plasmids were transfected into miR-181a-5p-silenced or control HGC-27 cells, respectively. (A-B) In vitro cell colony formation assays were performed in MKN-28 and HGC-27 cells. (C-D) In vitro transwell invasion assays were used to evaluate whether the overexpression or repression of RASSF6 could partially counteract the effects of up- or down-regulation of miR-181a-5p on GC cells' invasion ability. (E-F) Peritoneal dissemination metastatic assays were used to determine whether miR-181a-5p enhanced or inhibited the peritoneal metastasis of MKN-28 and HGC-27 cells by overexpressing or repressing RASSF6 expression in nude mice, respectively. (n = 3/group, *p < 0.05, **p < 0.01, ***p < 0.001).
Fig. 8. Ectopic expression of miR-181a-5p predicted poor survival in GC patients. (A) Expression of miR-181a-5p in the TCGA gastric cancer RNAseq dataset (normal n = 42, tumour n = 476). (B) Expression of miR-181a-5p in the Shanghai General Hospital dataset (normal n = 260, tumour n = 260). (C) Kaplan–Meier curves for recurrence free survival (RFS) of GC patients who had both the recurrence free survival and overall survival data in the TCGA dataset (n = 163). (D–F) Kaplan–Meier analyses for the Shanghai General Hospital dataset. (D) High expression of miR-181a-5p predicted short (5-year) RFS in patients, compared with those who expressed lower levels of miR-181a-5p. (E) Overall survival in patients without tumour recurrence. (F) Overall survival in patients with tumour recurrence. High expression of miR-181a-5p predicted the worst survival for the patients with recurrent tumours.
induce EMT in ovarian cancers [20]. High miR-181a-5p levels were also found to be involved in chondrosarcoma [21]. In addition, miR-181a-5p inhibited autophagy in the mammary epithelial cell line MCF10A [22]. Accordingly, the roles of miR-181a-5p in different diseases were different. It will be beneficial to use this specific miRNA to evaluate the prognosis of GC by investigating the function of miR-181a-5p in GC.

High proliferative, invasive and metastatic abilities are regarded as high risk factors of poor prognosis in cancers [23]. In this study, overexpression of miR-181a-5p also promoted GC cell proliferation and colony formation as well as induced the transition to the S-phase of the cell cycle and increased the expression of cell cycle-associated proteins in vitro. These results were consistent with the findings of other studies, indicating that miR-181a-5p enhanced GC cells proliferation [24]. The tumour xenograft assay in nude mice further supported the hypothesis that upregulation of miR-181a-5p accelerated tumour growth, as evidenced by the generation of larger xenografts and strong Ki-67 staining. Moreover, high miR-181a-5p expression enhanced GC cell wound healing, invasion abilities, and peritoneal dissemination of metastasis as well as upregulated the levels of invasive and metastatic proteins (MMP2 and MMP9) in vitro and in vivo. An increasing number of investigations has demonstrated that EMT plays significant effects in the progression of cancers and usually occurs by the loss of the epithelial phenotype and gain of mesenchymal characteristics [25]. In our study, overexpression of miR-181a-5p was associated with the low expression of the epithelial maker E-cadherin and high expression of the mesenchymal marker N-cadherin, both at the mRNA and protein levels, in vitro and in vivo. Similar effects on EMT have been reported in ovarian cancers [20]. Consequently, our study determined that miR-181a-5p was an onco-miRNA promoting cell proliferation, metastasis and EMT in GC.

As a member of the Ras-associated domain family, loss of RASSF6 expression was involved in Ras, P53, NFkB, MAPK and Hippo dependent bio-functions, such as, apoptosis, autophagy and invasion, which are closely correlated with cancer development [17,26,27]. In clear cell renal cell carcinoma, RASSF6 induced p21-dependent cell cycle arrest and promoted cell apoptosis via activation of the SAPK/JNK pathway [15]. In pancreatic ductal adenocarcinoma, low RASSF6 was positively associated with advanced T-stage and perineural invasion [28]. In melanoma, reduced expression of RASSF6 was positively associated with advanced T-stage and perineural invasion, which are closely correlated with cancer development [10]. In our study, overexpression of miR-181a-5p also promoted GC cell proliferation, metastasis and EMT in GC. miR-181a-5p inhibited RASSF6 expression by directly targeting its 3’UTR. Moreover, a multi-pathway reporter array demonstrated that miR-181a-5p, by repressing RASSF6, could activate the MAPK pathway in GC cells. miR-181a-5p-mediated tumour promoting roles could be partially impeded by an increase in RASSF6 expression. Clinically, miR-181a-5p was also inversely correlated with RASSF6 expression. Interestingly, miR-181a-5p alone could not predict the OS in the non-recurrent group. Meanwhile, patients showing high miR-181a-5p together with low RASSF6 had the worst OS. These findings highlighted that miR-181a-5p acted as an onco-miRNA through inhibition of RASSF6 in GC.

In conclusion, our study revealed the critical roles of the miR-181a-5p-mediated RASSF6/MAPK signalling pathway on the tumorigenesis and development of GC. This study not only provides new insight into the mechanisms elucidating the loss RASSF6 in GC but also suggests that miR-181a-5p is a marker of poor prognosis in GC patients.

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