MiR-19a/b modulate the metastasis of gastric cancer cells by targeting the tumour suppressor MXD1

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The microRNAs 19a and 19b, hereafter collectively referred to as miR-19a/b, were recognised to be the most important miRNAs in the oncomiRs–miR-17-92 cluster. However, the exact roles of miR-19a/b in cancers have not been elucidated. In the present study, miR-19a/b was found to be over-expressed in gastric cancer tissues and significantly associated with the patients’ metastasis of gastric cancer. Using gain or loss-of-function in *in vitro* and *in vivo* experiments, a pro-metastatic function of miR-19a/b was observed in gastric cancer. Furthermore, reporter gene assay and western blot showed that MXD1 is a direct target of miR-19a/b. Functional assays showed that not only MXD1 had an opposite effect to miR-19a/b in the regulation of gastric cancer cell migration, invasion and metastasis through targeting the antagonist of c-Myc – MXD1, implicating a novel mechanism for the malignant phenotypes of gastric cancer.

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MicroRNAs (miRNAs) are small, non-coding RNAs 19–24 nucleotides in length that act in association with the RNA-induced silencing complex to regulate expression of multiple genes by either inducing translational silencing or causing degradation of the mRNA of the targeted genes.1,2 It has been firmly recognised that miRNAs regulate many key cellular processes, such as cell growth, cell cycle, differentiation and cell death. Understanding miRNA functions and transcriptional regulatory circuits requires elucidation of the molecular pathways that are responsible for specific biological phenomenon through the integration of transcription factors, miRNAs and the direct targets of miRNAs.3

More recently, miRNAs have emerged as oncogenes or tumour suppressors4 that act as central players in networks that establish regulatory circuits in cancers. The miR-17-92 cluster, one of the miRNA clusters transcriptionally activated by the proto-oncogene c-Myc,5 first attracted attention following a series of observations that linked these miRNAs to cancer pathogenesis; the miR-17-92 cluster was shown to be over-expressed in many cancers, including lung, colon and breast cancer and neuroblastoma.6–9 An important role for these miRNAs in the regulatory circuit that controls cellular life and the cell cycle further illustrates the contributions of these miRNAs to tumorigenesis. Specifically, the transcriptional activators E2F1 and E2F3 can directly activate the transcription of miR-17-92, and, in turn, E2F1, E2F2 and E2F3 are among the first experimentally verified targets of these miRNAs.10

Although the miR-17-92 cluster members have been established as oncomiRs, their oncogenic contributions in cancers have not been assigned. The miR-17-92 cluster contains six members: miR-19a/b, miR-17/20a, miR-18a and miR-92a. We chose miR-19a/b for the present study because they were recently identified to be the most important miRNAs in this cluster,11,12 and we observed a pro-metastatic function of miR-19a/b in our primary studies. Expression of miR-19a/b was previously found to be correlated with lymph node metastasis in non-small cell lung cancer patients,13 however, the exact roles of miR-19a/b in cancer metastasis have not been elucidated. In the present study, we aimed at exploring the relationships between miR-19a/b and metastasis, and the potential mechanisms. Using gain and loss-of-function assays, a pro-metastatic function of miR-19a/b was observed. Furthermore, the tumour suppressor MXD1 was demonstrated to be a direct target of miR-19a/b. Our findings provide mechanistic insight into the function of miR-19a/b and have important implications for understanding gastroenterological disorders resulting from altered regulation of miRNA pathways.

Results

Expression of miR-19a/b was associated with metastasis in gastric cancer patients. miR-19a/b were previously regarded as oncomiRs and were detected to regulate the self-renewal of gastric cancer stem cells in our department

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Abbreviations: miRNA, microRNA; MXD1, MAX dimerisation protein 1; RISC, RNA-induced silencing complex; E2F1, E2F transcription factor 1; E2F2, E2F transcription factor 2; E2F3, E2F transcription factor 3; HR, hazard ratio; EGFP, enhanced green fluorescent protein; 3’UTR, 3’ untranslated region; AS, antisense; MAX, MYC associated factor X

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We firstly tested the expression of miR-19a/b in gastric cancer tissues by real-time PCR using RNAs from 141 patients who underwent gastrectomy and were followed up at Xijing Hospital during 2006–2008. We used a log$_2^{-\Delta\Delta CT}$ analysis to statistically describe the expression levels of miR-19a/b. As shown in Figures 1a and b, the results verified that the expression of miR-19a (A) or miR-19b (B) was higher in 10 of 15 or 10 of 15 randomly selected human gastric cancer tissues, respectively, compared with the adjacent normal tissues. Interestingly, there was 13 out of 15 gastric cancer samples with either miR-19a or miR-19b overexpression from the results of the real-time PCR (Figures 1a and b). Correlations between miR-19a/b expression levels and clinicopathologic characteristics of gastric cancer are summarised in Table 1. A statistically significant association between miR-19a/b expression levels and metastasis of gastric cancer patients was observed in this study. The expression of miR-19a or miR-19b in gastric cancer patients did not correlate with age, gender or cell differentiation, but correlated with remote metastasis of gastric cancer patients ($P<0.001$). The expression of miR-19a was also significantly associated with TNM stage and lymph node metastasis ($P<0.001$).

To examine whether the expression levels of miR-19a/b were associated with survival in the above gastric cancer patients, we ranked the patients on the basis of the relative

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**Figure 1**  Expression of miR-19a/b in gastric cancer tissues and their association with clinicopathologic characteristics. (a and b) Real-time PCR shows the expression of miR-19a (a) and miR-19b (b) in 15 randomly selected gastric cancer samples compared with the adjacent normal tissues (**$P<0.01$, *$P<0.05$). Each experiment was independently repeated at least three times. Error bars correspond to the mean $\pm$ S.D. (c) Gastric cancer samples were divided into two groups, according to the median expression of miR-19a (median $=2.99$). Cases with levels of miR-19a below the mean were considered miR-19a low expressers ($n=70$), and those with levels of miR-19a above the mean were considered miR-19a high expressers ($n=70$). (d) Kaplan–Meier survival curve and log-rank test for gastric cancer patients between high and low miR-19a expressers to evaluate the relationship with patient survival.
expression levels obtained via real-time PCR and divided them into high-expression group and low-expression group using the 50th percentile (median) as the cut-off point (Figure 1c). HR from the univariate Cox regression analysis showed that the TNM stage and the expression of miR-19a was correlated with death from any cause (Figure 1d, \( P < 0.001 \)). A multivariate Cox regression analysis identified the level of miR-19a as an independent factor that was associated with overall survival (Supplementary Table 1).

### Table 1: Association of miR-19a/b expression with clinicopathologic characteristics

| Variables                        | No. of cases (%) | Expression of miR-19a | Expression of miR-19b |
|----------------------------------|------------------|-----------------------|-----------------------|
|                                  |                  | Mean ± S.D.           | P-value               | Mean ± S.D. | P-value |
| Age (years)                      |                  |                       |                       |
| ≥60                              | 72 (51%)         | 3.93 ± 3.03           | 0.57                  | 1.55 ± 1.03 | 0.44     |
| <60                              | 69 (49%)         | 3.64 ± 3.04           |                       | 1.40 ± 1.22 |          |
| Gender                           |                  |                       |                       |
| Male                             | 103 (73%)        | 3.84 ± 3.05           | 0.74                  | 1.44 ± 1.04 | 0.54     |
| Female                           | 38 (27%)         | 3.65 ± 2.99           |                       | 1.57 ± 1.33 |          |
| Degree of differentiation        |                  |                       |                       |
| Well and moderately differentiated| 46 (33%)         | 3.19 ± 2.89           | 0.11                  | 1.45 ± 0.82 | 0.83     |
| Poorly differentiated             | 95 (67%)         | 4.08 ± 3.07           |                       | 1.49 ± 1.25 |          |
| TNM stage                        |                  |                       |                       |
| Stage I/II                       | 67 (48%)         | 2.26 ± 2.49           | <0.001                | 1.31 ± 0.99 | 0.09     |
| Stage III/IV                     | 74 (52%)         | 5.18 ± 2.81           |                       | 1.63 ± 1.22 |          |
| Lymph node metastasis            |                  |                       |                       |
| Yes                              | 86 (61%)         | 4.91 ± 2.92           | <0.001                | 1.57 ± 1.20 | 0.20     |
| No                               | 55 (39%)         | 2.19 ± 2.48           |                       | 1.32 ± 0.98 |          |
| Remote metastasis                |                  |                       |                       |
| Yes                              | 21 (15%)         | 6.96 ± 2.50           | <0.001                | 2.20 ± 1.90 | <0.001   |
| No                               | 120 (85%)        | 3.23 ± 2.77           |                       | 1.35 ± 0.88 |          |

**Stable overexpression of miR-19a/b miRNAs promoted gastric cancer cell migration and invasion in vitro.** Because miR-19a/b were over-expressed in gastric cancer tissues compared with normal tissues and their expression was associated with the metastasis of gastric cancer patients, we speculated that the overexpression of miR-19a/b might promote the migratory and invasive abilities of gastric cancer cells. To test the metastatic ability of miR-19a/b, we constructed an EGFP-expressing lentivirus with miR-19a/b precursors or a negative control small RNA and infected them into gastric cancer cell lines SGC7901 and MKN28. The EGFP-positive cells were detected using fluorescence microscopy and were sorted using a FACSM machine as indicated in Supplementary Figures 1A and B. After sorting for EGFP-positive cells, the overexpression of mature miR-19a/b was confirmed by real-time PCR as shown in Figures 2a and b. The SGC7901 and MKN28 cells infected with lent-miR-19a/b showed pro-migratory ability compared with the lenti-NC infection group (Figure 2c). Similar results were observed in cell invasion assays as indicated in Figure 2d.

To avoid the cross reaction of miR-19a and miR-19b, we performed real-time PCR of miR-19a/b in miR-19a and miR-19b-stable over-expressing cells. As shown in Supplementary Figure 3A, miR-19b expression did not increase in miR-19a-stable over-expressing cells compared with the NC group (\( P > 0.05 \)). Also, the miR-19a expression did not change in miR-19b over-expressing cells compared with the NC group as shown in Supplementary Figure 3B (\( P > 0.05 \)).

**Transient transfection of miR-19a/b precursors and inhibitors regulated gastric cancer cell migration and invasion in vitro.** To avoid the experimental artefacts with regard to cellular changes induced by the stable infection of the lentiviral constructs, we further purchased commercial miR-19a/b precursors and inhibitors from Applied Biosystems (Invitrogen, Carlsbad, CA, USA). We performed transient transfection of SGC7901 and MKN28 gastric cancer cell lines using a siPORT NeoFX Transfection Agent (Invitrogen) with miR-19a/b precursors and inhibitors. The transient transfection efficiency was detected by real-time PCR (Supplementary Figures 2A and B, Figures 3a and b). Consistent with the stable transfection results, the transient transfection of miR-19a or miR-19b precursors significantly increased the migration and invasion abilities of gastric cancer cells (Supplementary Figures 2C and D), while the inhibitors remarkably impeded cell migration and invasion in vitro (Figures 3c and d).

miR-19a/b stably over-expressing cells were prone to metastasise in nude mice. To test the in vivo relationships between miR-19a/b and metastasis, we performed a caudal vein injection using cells stably expressing miR-19a/b in BALB/C nude mice. To facilitate the detection of metastasis, a luciferase-labelled SGC7901Luc cell line established in our lab was infected with lent-miR-19a, lent-miR-19b miRNAs or the negative control lentivirus (cells named SGC7901Luc-19a, SGC7901Luc-19b or SGC7901Luc-NC, respectively). The luciferase signals of SGC7901Luc cells and the
miR-19a/b miRNAs were stably over-expressed in gastric cancer cell lines SGC7901 and MKN28 by lentiviral infection. Overexpression of miR-19a/b promoted gastric cancer cell migration and invasion. (a and b) Successful stable overexpression of miR-19a/b in gastric cancer cell lines SGC7901 and MKN28 was confirmed by real-time PCR. Fold change was used to calculate each of the values of miR-19a/b relative to the negative control. Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (** P<0.01, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (c) Representative images of migrated cells on transwell plates that were originally plated with 10^5 cells; the number of migrated cells is indicated (*** P<0.01, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (d) Representative images show cells invaded through transwell coated with matrigel and the invaded cell numbers. In the upper chambers, 5 x 10^4 cells were plated and incubated for 24 h at 37 °C. Cells invaded through to the downside of the membrane, were fixed with 100% methanol and stained with 0.2% crystal violet (*** P<0.01 compared with the NC group, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D.

Figure 2 miR-19a/b miRNAs were stably over-expressed in gastric cancer cell lines SGC7901 and MKN28 by lentiviral infection. Overexpression of miR-19a/b promoted gastric cancer cell migration and invasion. (a and b) Successful stable overexpression of miR-19a/b in gastric cancer cell lines SGC7901 and MKN28 was confirmed by real-time PCR. Fold change was used to calculate each of the values of miR-19a/b relative to the negative control. Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (c) Representative images of migrated cells on transwell plates that were originally plated with 10^5 cells; the number of migrated cells is indicated (** ** ** ** P<0.01, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (d) Representative images show cells invaded through transwell coated with matrigel and the invaded cell numbers. In the upper chambers, 5 x 10^4 cells were plated and incubated for 24 h at 37 °C. Cells invaded through to the downside of the membrane, were fixed with 100% methanol and stained with 0.2% crystal violet (** P<0.01 compared with the NC group, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D.

miR-19a/b miRNAs targeted MXD1 transcript by binding the 3' UTR of MXD1 transcripts. *In silico* analysis using miRanda software (http://www.microrna.org/microrna/home.do) showed that the 3'-UTR of human MXD1 contains two conserved putative target sites for miR-19a/b miRNAs (Figure 5a). To validate these target sites, the 3'-UTR of human MXD1 was amplified and inserted in both orientations downstream of the luciferase gene in the pGL3-control vector, generating sense (S) and antisense (AS) constructs, collectively referred to as Luc-1 in Figure 5a. Two shorter clones (Luc-2 and Luc-3) were prepared similarly (Figure 5a). The transfection of SGC7901 cells with miR-19a or miR-19b precursors significantly decreased the expression of Luc-1S while having no effect on Luc-1AS expression (Figure 5b). In contrast, no affect was observed in cells transfected with miR-150, in accordance with the fact that the MXD1 3'-UTR does not contain a miR-150 target site (Figure 5b). The expression of Luc-2S was also reduced by either miR-19a or miR-19b precursors but not miR-150 (Figure 5c). Furthermore, miR-19a and miR-19b targeted Luc-3S but not Luc-3AS, as indicated in Figure 5d.
Overexpression of MXD1 without 3' UTR-impaired miR-19a/b effects. To determine the downregulating effects of miR-19a/b at the protein level, we performed western blotting assays using an anti-MXD1 antibody. As expected, the transient transfection of SGC7901 cells with pre-miR-19a or pre-miR-19b decreased MXD1 levels (Figure 5e), while cells transiently transfected with miR-19a or miR-19b inhibitors showed increased MXD1 levels, which suggest that miR-19a/b miRNAs regulate MXD1 expression in vivo at the post-transcriptional level.

To further confirm that MXD1 is a target of miR-19a and miR-19b, we performed real-time PCR using RNAs from the 15 paired malignant and normal tissues. As shown in Figures 6a and b, expression of MXD1 adversely correlated with both the expression of miR-19a (A, \( P = 0.001 \)) and miR-19b (B, \( P = 0.009 \)).

To further prove that MXD1 is the major player downstream of miR-19a/b, we performed migration assay using cells transfected with pre-NC, pre-miR-19a, pre-miR-19b and MXD1 without 3' UTR, pre-miR-19b, pre-miR-19b and MXD1 without 3' UTR. The results showed that cotransfection of MXD1 without 3' UTR, restricted the effects of pre-miR-19a or pre-miR-19b transfection alone as indicated in Figures 6c and d. In another word, the overexpression of miR-19a/b was rescued by overexpression of MXD1 without 3' UTR.

Overexpression of MXD1 repressed c-Myc and miR-19a/b levels and impeded the malignant phenotypes of gastric cancer cells. It was previously reported that MXD1 can compete with c-Myc for interaction with MAX and has an opposite role to c-Myc in the regulation of the growth and function in gastric cancer cells. To test c-Myc expression and MXD1 function in gastric cancer cells when MXD1 is overexpressed, we constructed an HA-tagged MXD1. As indicated in Figure 7a, western blot showed that the c-Myc level was reduced in cells transfected with HA-MXD1. In vitro migration and invasion assays (Figures 7b and c) showed that HA-MXD1 impeded migration and invasion abilities of gastric cancer cells, while si-MXD1 promoted migration and invasion compared with the vector control group. MTT assays indicated that MXD1 decreased the growth speed of SGC7901 cells, while si-MXD1 promoted proliferation in...
gastric cancer cells (Figure 7d). Our results demonstrated that MXD1 has an antimigratory, anti-invasive and antiproliferative function in gastric cancer. Moreover, the present study demonstrated that as the target gene of the oncomiRs miR-19a/b, MXD1 has a tumour-suppressive effect in gastric cancer cells.

To test whether miR-19a and miR-19b levels were regulated by MXD1, we performed real-time PCR on MXD1 over-expressing cells or cells transfected with MXD1 siRNAs (si-MXD1). As shown in Figures 7e and f, in MXD1 over-expressing cells, the expression levels of miR-19a and miR-19b were two or threefold lower than in the vector control group (Figure 7f). These results demonstrated that although miR-19a/b can downregulate MXD1 expression by binding its 3'UTR, overexpression of MXD1 can also reduce miR-19a/b expression levels, indicating a potential miRNA target gene self-regulatory loop between miR-19a/b and MXD1.

**Discussion**

The human miR-17-92 cluster is located on 13q31.3, a frequently amplified locus in colorectal cancers and lymphomas. The miR-17-92 cluster members, including miR-17-20a, miR-19a/b, miR-18a and miR-92a, were identified to be over-expressed in many cancers, including breast,6 lung,7 pancreatic,14 colon8 and gastric cancer.15,16 Each member of the cluster is predicted to regulate a variety of different cellular targets; however, the functions of miRNAs in this cluster were still unclear. He L et al.17 originally identified the miR-17-92 cluster as a potential human oncogene in B-cell lymphomas. Later, O Donnell et al.5 demonstrated the transcriptional activation of the miR-17-92 cluster by the proto-oncogene c-Myc, thus establishing a potential role of this cluster in cell proliferation, growth and apoptosis. Recently, Mu et al.11 identified miR-19a/b as the most important miRNAs of this cluster. Using deletion-reintroduction mouse models, they found that reintroduction of miR-19a/b rescued oncogenicity but not to the same extent as reintroduction of the entire cluster, indicating the pro-tumorigenic activity within this cluster. Using gain-of-function models, Olive et al.12 also found that overexpression of the full cluster without miR-92 enhanced tumorigenesis, but when miR-19a and miR-19b were lost from the cluster, tumorigenesis was not enhanced, indicating the important function of miR-19 in tumorigenesis.

Although miR-19a and miR-19b were found to be the most significant miRNAs of the miR-17-92 cluster, and were
reported to have a broad and significant roles in normal or abnormal cells of human body, the exact function and the potential mechanisms of them in gastric cancer has not been fully clarified. We have previously found that the expression of the miR-17-92 cluster members were gradually changed during the differentiation of gastric cancer stem cells, among the six members, miR-19b/20a/92a can regulate the self-renewal of gastric cancer stem cells through activating the Wnt-β-catenin signalling. Further study found that miR-19a/b could sustain the multi-drug resistance of gastric cancer cells. In the present study, we first observed that miR-19a/b was over-expressed in most of the randomly selected gastric cancer samples and overexpression of miR-19a and miR-19b was significantly associated with metastasis in gastric cancer patients. Furthermore, in vivo and in vitro experiments were performed to test the relationships between miR-19a/b miRNAs and metastasis in gastric cancer using both stable and transient miR-19a/b expressing cells. Moreover, a pro-migratory, pro-invasive and pro-metastatic effect was observed in this study using in vitro and in vivo metastatic assays, which was consistent with the previous studies that the miR-17-92 cluster acts as oncomiRs in cancers.

Figure 5 MXD1 transcripts were direct targets of miR-17-92 miRNAs. (a) Schematic representation of MXD1 3′-UTR constructs in pGL3-Control (Luc-1,2,3). Conserved putative target sites for miR-19a/b miRNAs were indicated. 19a, 19b and 19a/b in the figures mean miR-19a, miR-19b and miR-19a/b, respectively. (b–d) Luciferase assays were performed with Luc-1(B), Luc-2(C) and Luc-3(D) as indicated, both in sense (S) and antisense (AS) orientations. Bars indicate the ratios of the firefly luciferase (normalised to Renilla luciferase) activity measured following transfection with miR-19a/b pre-miRNAs to that obtained following transfection with the pre-miR control (pre-NC) for the same construct (**P<0.01, compared with the AS group, t-test). Each experiment was repeated at least three times. Error bars correspond to the mean ± S.D. (e) Western blot showing the changes in MXD1 protein levels after transient transfection of miR-19a/b precursors or inhibitors.
sequences of MXD1. Reintroducing MXD1 in cells impeded the migration, invasion and proliferation of gastric cancer cells and reduced the expression of miR-19a/b, while downregulating MXD1 by si-MXD1 promoted gastric cancer migration and invasion and increased miR-19a/b levels. These results indicate a unique mechanism by which a miRNA acts on a specific target within its multiple target pool to modulate specific responses.

Recently, miR-17-92 members have emerged as central players in networks that establish regulatory feedback circuits. Additionally, the targeted genes of this cluster are involved in distinct pathways so that the targeted gene may exert opposing effects. Several previous reports confirmed that the E2F transcription factor family members can induce the expression of miR-17-92, and two of these family members are direct targets of miR-17-92, which generate a negative feedback loop aimed at restricting miR-17-92 transcription levels. In another report, Tili et al. discovered that GAM reduces the transcriptional activation of the miR-17-92 promoter, downregulates miR-17-92 expression and limits the activation of genes related to the TGF-β canonical pathway. In contrast, TGF-β reduces GAM transcript levels while differentially upregulating miR-17-92 expression. In turn, miR-17-92 targeted GAM transcripts, thus establishing another auto-regulatory feedback loop. In the present study, despite the relationships between miR-19a/b and MXD1, we also found that c-Myc, the transactivator of miR-19a/b, can be reduced by introducing MXD1 into gastric cancer cells. Thus, the direct link between miR-19a/b miRNAs and the c-Myc antagonist gene MXD1 demonstrated a positive feedback loop between c-Myc, miR-19a/b and MXD1 that may be useful in reducing the opposite effects. Thus, auto-regulation contributes to the sustained induction of malignancies in gastric cancer.
As a last remark, the emergence of cancer coincides with a failure in regulating gene duplication, gene transcription and/or the levels of signalling molecules involved in regular metabolism. Therefore, it is possible that a new level of molecular complexity in tumours is generated by the development of robust genetic circuitries for the maintenance of cellular dysfunction and the prohibition of the organism to adapt to changing environments. MXD1, c-Myc and the miR-19a/b miRNAs have all been shown to increase in cancers; therefore, it is not surprising that they may be implicated in the control of cancer cell progression and tumour development and that they may interact with each other in different ways. Our study implied that the positive autoregulatory network among miR-19a/b, MXD1 and c-Myc might be important in preserving the expression levels of oncogenic miRNAs and thus might provide a new viewpoint for the mechanism of the metastasis in gastric cancer.

Materials and Methods

Ethics statement. For the analysed tissue specimens, all patients gave informed consent to use excess pathological specimens for research purposes. The protocols employed in this study were approved by the hospital’s Protection of Human Subjects Committee. The use of human tissues was approved by the institutional review board of the Fourth Military Medical University and conformed to the Helsinki Declaration, and to the local legislation. Patients offering samples for the study signed informed consent forms. In the animal experiments, all procedures for animal experimentation were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Experimental Animal centre of the Fourth Military Medical University. The approval ID for using the animals was No.12039 from Experimental Animal Centre of the Fourth Military Medical University.

Cell culture and reagents. The human gastric adenocarcinoma cell line SGC7901 was obtained from the Academy of Military Medical Science, and MKN28 was obtained from the Shanghai Cell Bank (Shanghai, China). These two cell lines were preserved in our institute. SGC7901Luc cells stably expressing firefly luciferase were generated and preserved in our lab. All of the cells were
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grown in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS) at 37 °C with 5% CO2 in a humidified incubator (Forma Scientific, Marietta, OH, USA). The precursors (pre-miRs) and inhibitors (miR-inhs) of the miRNAs miR-19a and miR-19b were obtained from Applied Biosystems (Invitrogen).

Construction of miR-19a and miR-19b lentivirus. For miR-19a and miR-19b sequences, two PCR primers were annealed to get the inserted sequences. The lentiviral vector pGCSil-H1 was double digested, purified and directly connected with the annealed products. The products were further transformed to competent bacterial cells for the growth of colonies, which were identified using PCR to get the positive clones. Positive clones identified by PCR were sequenced and analysed. Right clones were considered to be successfully constructed miR-19a and miR-19b plasmid lentiviral vectors. After ultrapure extraction to remove the endotoxin, the vectors were packaged with lentivirus into 293T cells using LipoFacetamine 2000 (Invitrogen). Lentivirus was harvested and concentrated from cells and then titre was detected using GFP expressing strength.

Lentivirus miR-19a/b infection and stable cells. The lentiviral system with EGFP-expressing miR-19a (lenti-miR-19a) and miR-19b (lenti-miR-19b) and the negative control lentivector (lenti-NC) were purchased from Genechem (Shanghai Genechem Co., Ltd, Shanghai, China). Gastric cancer cell lines SGC7901, SGC7901Luc and MKN28 were infected with lentiviral vector, lenti-miR-19a/b or lenti-NC, according to the manufacturer’s instructions, and stable cells were isolated by flow cytometry to sort EGFP-positive cells using flow cytometry.

Cell migration and invasion assays. Transwell migration and invasion assays were performed as described before.30 The invasion assay was performed with Matrigel (BD Biosciences, Sparks, MD, USA) coated on the upper surface of the transwell chamber (Corning, Lowell, MA, USA). Cells that invaded or migrated through the membrane were fixed with methanol and stained with crystal violet. Photographs of four randomly selected fields of the fixed cells were taken and counted. Each experiment was repeated independently three times.

Animals and the in vivo metastasis assay. BALB/C nude mice at 4–6 weeks of age were handled using the best humane practices and cared for in accordance with NIH Animal Care Institutional Guidelines in the Experiment Animal Centre of the Fourth Military Medical University (X’an, Shaanxi Province, China). Firefly luciferase-labelled SGC7901 cells—SGC7901Luc were infected with lenti-miR-19a, lenti-miR-19b or lenti-NC, respectively, as described above, and stable clones were isolated. For in vivo metastasis assay, 2 × 105 cells were injected into the caudal veins of each nude mouse. Each group (miR-19a group, miR-19b group and NC group) contained six mice. After injection, bioluminescent signals were detected twice a week by the IVIS 100 Imaging System (Xenogen). Five minutes before imaging, the mice were injected intraperitoneally with 100 mg/kg D-luciferin. The mice were sacrificed seven weeks after injection of gastric cancer cells. For histological analysis, the lungs and livers of nude mice were harvested and fixed in 10% formalin before paraffin embedding, and then sectioned and stained with H&E staining.

Reporter gene assay. For the reporter gene assay, cells were plated in 12-well plates and were transfected with 2 μg of MxD1 3'-UTR luciferase reporter plasmids (S) or the MxD1 3'-UTR antisense mutant (AS) and the empty pGL3-Control vector (Promega Biotech Co., Ltd, Beijing, China) using lipofection 2000 (Invitrogen). Cells were also cotransfected with the pre-miRNAs (150 nM, Applied Biosystems, Invitrogen). Assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay system (Promega Biotech Co., Ltd). Firefly luciferase activities were normalised to Renilla luciferase activities. A miRNA precursor molecule control from Applied Biosystems (Invitrogen). Assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay system (Promega Biotech Co., Ltd). Firefly luciferase activities were normalised to Renilla luciferase activities. A miRNA precursor molecule control from Applied Biosystems (Invitrogen). 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Statistical analysis. Continuous variables were compared by Student’s t-test or ANOVA test. If the test result of the homogeneity of variances between the groups was significant, the Mann–Whitney test was appropriately adopted. The χ2-test or Fisher’s test was used for categorical variables. The independent predictors of survival were calculated using the Cox regression model. The covariates incorporated into the multivariate analysis were the variables reaching P < 0.05 in an univariate analysis. Cumulative survival was assessed with Kaplan–Meier curves and compared using the log-rank test. Two-tailed P-values < 0.05 were considered statistically significant (”P < 0.05; **P < 0.01). All statistical analyses were conducted using SPSS software, version 14.0 (SPSS Inc., Chicago, IL, USA).

Conflict of Interest

The authors declare no conflict of interest.

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1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281–297.
2. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215–233.
3. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol Cell 2010; 38: 493–506.
4. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857–866.
5. D’Ondell KA, Wentzel EA, Zeller KT, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005; 435: 859–863.
10. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell 2009; 138: 217–222.

11. Mu P, Han YC, Betel D, Yao E, Squatrito M, Ogrodowski P et al. Genetic dissection of the miR-17–92 cluster of microRNAs in Myc-induced B-cell lymphomas. Genes Dev 2009; 23: 2806–2811.

12. Olive V, Bennett MJ, Walker JC, Ma C, Jiang I, Cordon-Cardo C et al. miR-19 is a key oncogenic component of miR-17-92. Genes Dev 2009; 23: 2839–2849.

13. Lin Q, Chen T, Lin G, Lin J, Chen G, Guo L. Serum miR-19a expression correlates with worse prognosis of patients with non-small cell lung cancer. J Surg Oncol 2013; 107: 767–771.

14. Volinia S, Calin GA, Liu CG, Amba S, Cimmino A, Petrocca F et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 2006; 103: 2257–2261.

15. Yao Y, Suo AL, Li ZF, Liu LY, Tian T, Li Z et al. MicroRNA profiling of human gastric cancer. Mol Med Report 2009; 2: 963–970.

16. Guo J, Xiao B, Huan R, Jiang Z, Meng D et al. Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. J Gastroenterol Hepatol 2009; 24: 652–657.

17. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S et al. A microRNA polycistron as a potential human oncogene. Nature 2005; 435: 828–833.

18. Song DW, Ryu JY, Kim JO, Kwon EJ, Kim do H. The miR-19a/b family positively regulates cardiomycocyte hypertrophy by targeting atrogin-1 and MuRF-1. Biochem J 2014; 457: 151–162.

19. Xu XM, Wang XB, Chen MM, Liu T, Li YX, Jia WH et al. MicroRNA-19a and -19b regulate cervical carcinoma cell proliferation and invasion by targeting CUL5. Cancer Lett 2012; 322: 148–158.