Abstract. microRNAs (miRs) serve critical roles in tumor progression. Low expression of miR-125a in gastric carcinoma (GC) may promote tumor development. In the present study, low expression of miR-125a was confirmed in cancer tissues using The Cancer Genome Atlas database. Additionally, the expression and clinical significance of miR-125a-5p was investigated using reverse transcription-quantitative PCR in 150 cases of GC. The results of the present study demonstrated that the level of miR-125a-5p expression was decreased in GC biopsies compared with that in matched adjacent normal tissues. Low expression of miR-125a-5p was associated with increased tumor diameter, high Ki67 expression and poor overall survival of patients with GC. Multivariate survival analysis demonstrated that low miR-125a-5p expression may be used as an independent prognostic factor for patients with GC. However, no effects on the cell viability in a Cell Counting kit-8 assay, and cell migration and invasion in Transwell assays were detected in response to treatment using miR-125a-5p mimics or inhibitors in vitro. Therefore, the results of the present study provide evidence that low expression of miR-125a-5p may be associated with a poor prognosis, suggesting its value as a tumor biomarker for patients with GC.

Introduction

Gastric cancer (GC) is the third leading cause of cancer-associated mortality worldwide (1). According to the National Central Cancer Registry of China, GC is the second most common cause of cancer-associated mortality in China, accounting for ~498,000 deaths in 2015 (2). The majority of patients with GC (>80%) are diagnosed at an advanced stage, therefore leading to 5-year survival rates of <25% (3).

MicroRNAs (miRNAs/miRs) are short non-coding RNA molecules of 18-25 nucleotides in length. They serve important roles in the initiation and progression of human cancer and may be used as promising therapeutic targets (4). It has been reported that several miRNAs are involved in cancer progression and diagnosis, and are associated with patient survival or drug-resistance in GC (5-9). For example, circulating levels of miR-627, miR-629 and miR-652 may be used as promising classifiers for GC, and greatly enhance the feasibility of circulating miRNAs as non-invasive diagnostic markers (10). Downregulation of miR-491-5p is associated with larger tumor size, and promotes GC metastasis by regulating SNAIL family transcriptional repressor and fibroblast growth factor receptor 4 (11). A three-miRNA signature (miR-145-3p, miR-125b-5p and miR-99a-5p) is significantly associated with the survival of patients with GC (12). miR-17-5p may induce cisplatin-resistance of GC cells by modulating apoptosis via targeting p21 (13). Therefore, examining the expression profile of miRNAs in GC is important.

In the present study, expression of the miR-125a/let-7e/miR-99b cluster was analyzed using The Cancer Genome Atlas (TCGA) database; it was shown that miR-125a expression was decreased in GC tissues compared with in adjacent normal tissues. Additionally, the results showed that reduced miR-125a-5p expression was associated with high tumor diameter and poor survival of patients with GC. However, inhibitory effects of miR-125a-5p on cancer cell proliferation, migration and invasion were not identified in vitro.

Materials and methods

TCGA database. Stomach adenocarcinoma (STAD) miRNA expression data was downloaded. A total of 43 pairs of matched tumor and adjacent normal tissues were included within the TCGA-STAD dataset and were obtained using SPSS (version 18.0.0; SPSS, Inc.) software based on the sample IDs [criteria for each pair of matched samples: i) The first 13 characters of each sample ID were the same; and ii) the last two characters of each sample ID represented either primary tumor (code: 01) or solid tissue normal (code: 11) from the TCGA database (https://cancergenome.nih.gov/). Then, the normalized expression data (level 3) of the miR-125a/let-7e/miR-99b cluster according to miRNA-seq calculated using the log2 algorithm (tumor/normal) in the...
selected samples were obtained, and the statistical significance between tumor and adjacent normal tissues was evaluated using a paired t-test.

**Tissue specimens.** A total of 150 GC tissue specimens and 97 matched adjacent normal tissues were obtained from patients (mean age, 58.96±1.76 years; age range, 32-82 years; 109 males and 41 females) that underwent surgery at Peking University Cancer Hospital and Institute (Beijing, China) between December 2004 and October 2008. The following inclusion criteria were applied: i) Stomach adenocarcinoma; ii) without preoperative radiation or chemotherapy; and iii) with records of overall survival (OS) information. The present study was approved by the Ethics Committee of Peking University Cancer Hospital and Institute (no. 2017KT79). Written informed consent was obtained from all participants.

**Cell culture.** The NCI-N87, NUGC-3, SGC-7901, AGS, MGC-803 and BGC-823 human GC cell lines were obtained from the Cell Center of Peking Union Medical University (Beijing, China). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C with 5% CO₂. MGC-803 and BGC-823 human GC cell lines were obtained from all participants.

**Cell transfection with miR‑125a‑5p mimics or inhibitors.** miRNA mimics or inhibitors for miRabse accession no. MIMAT0000443 were synthesized by Guangzhou RiboBio Co., Ltd., and transiently transfected into 1x10⁵ cells at a 50 nmol/l concentration using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol for 24 h prior to subsequent experimentation. The mimics sequence (5'-UCCUCAGAGCCUUUAAAAGGUGA-3'), inhibitors sequence (5'-TCA CAGGUGUAAAGGTTCTACAGGA-3') and negative control sequence (NC; 5'-UUCUCGAACGUGUCAGUU-3') were all designed as chemically modified double strands.

**Reverse transcription‑quantitative PCR (RT‑qPCR).** Total RNA was isolated from tissues and cells using miRNeasy Mini kits (Qiagen, Inc.) according to the manufacturer's protocols. Poly(A) tails were added to total RNA 3'-ends (14,15). Subsequently, RNA was reverse transcribed with an oligodT-adaptor primer (5'-GCG AGC ACAGAATTAATACGCATCTATAGGTTTTTTTTTT TTVN-3') and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. The following primers were used: miR-125a-5p, forward 5'-TGGAGACCTTTTCACCAGTGTA-3' and reverse 5'-GCCGACACAGAATTAATACGCAC-3'; U6, forward 5'-CTCGCTTCGGCAGCGACCA-3' and reverse 5'-AAGCCT TCACGAAATTTCGCGTTGTA-3'. The expression levels of miRNA were evaluated using SYBR® Select Master Mix (Thermo Fisher Scientific, Inc.) and an ABI7500 fast System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. U6 was used as the reference gene. The expression level of miR-125a-5p was calculated using the 2^ΔΔCq method, whereby ΔCq=Cq (miR-125a-5p)-Cq (U6) (16,17).

**Cell viability assay.** A total of 5x10³ cells/well were seeded into 96-well plates and cultured for 24, 48, 72 and 96 h. Cell growth was evaluated using a Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Absorbance was measured at a wavelength of 450 nm using a microplate reader (iMark; Bio-Rad Laboratories, Inc.).

**Transwell migration and invasion assays.** Cells (1x10⁴) were seeded into the upper chambers of the Transwell plate, with or without Matrigel, in 100 µl RPMI-1640 medium containing 1% FBS. A total of 500 µl RPMI-1640 medium containing 10% FBS was placed in each lower chamber as a chemottractant. After a 24-h incubation at 37°C, migrated or invaded cells were fixed with 4% formaldehyde at room temperature for 5 min and stained with 1% crystal violet for 5 min at room temperature. The number of migrated or invasive cells was counted using a light microscope (magnification, x20) in five randomly-selected microscopic fields.

**Statistical analysis.** Data were analyzed using SPSS 13.0 software (SPSS, Inc.). The relevant data are expressed as the mean ± standard deviation from three independent experiments. Statistical significance was determined for two groups using a two-tailed Student's t-test, and for multiple comparisons using one-way ANOVA with a Bonferroni post hoc test. A Mann Whitney U test was used for the comparison of two independent groups as a nonparametric test on ranked data. A two-tailed χ² test was used to evaluate the association between miR-125a-5p expression and clinicopathological factors. OS was analyzed using the Kaplan-Meier method and a log-rank test. The cut-off value for miR-125a-5p expression was based on the receiver-operator curve (ROC) corresponding to the maximum Youden index. A univariate Cox analysis was performed followed by a multivariate Cox regression analysis including only variables which were significant in the univariate Cox analysis (18,19). Pearson's correlation analysis was conducted to determine the correlation between the level of miR-125a-5p expression and the migration or invasion ability in GC cell lines. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of miR-125a in GC tissues.** miR-125a/let-7e/miR-99b expression levels were analyzed in GC tissues based on data derived from the TCGA database. Even though the three miRNAs derive from the same precursor, only miR-125a exhibited low expression in GC tissues compared with adjacent normal tissues (Fig. 1A). There was no significant difference in the expression of let-7e or miR-99b between GC and matched adjacent normal tissues (Fig. 1B and C).

**Low expression of miR-125a-5p is associated with larger tumor diameter and poor survival of patients with GC.** To further confirm the expression profile of miR-125a-5p, RT-qPCR was performed using 97 pairs of matched tumor and adjacent normal tissues. The results indicated significantly
decreased expression levels of miR-125a-5p in GC tumor tissues compared with adjacent normal tissues (Mann Whitney U test; P=0.001; Fig. 2A). The expression level of miR-125a-5p was subsequently detected in 150 cases of patients with GC, and the association between miR-125a-5p expression and the clinicopathological parameters of patients with GC was evaluated. The average expression level of miR-125a-5p was decreased when the tumor diameter was ≥5 cm, compared with tumors of <5 cm diameter (1.491±2.929 vs. 1.778±2.278; P=0.0474; Fig. 2B). According to the cut-off value of the ROC curve corresponding to the maximum Youden index (Fig. 2C), patients were divided into high or low miR-125a expression groups. As shown in Table I, the number of patients with low miR-125a-5p expression was higher in if tumor size is ≥5 cm and Ki67 expression is high, indicating that low miR-125a-5p expression may be associated with tumor growth. However, there was no association between miR-125a-5p expression and other clinicopathological features, including sex, differentiation, TNM stage, lymph node metastasis, tumor embolus and distant metastasis (Table I). Nevertheless, the number of patients with low miR-125a-5p expression was higher in patients ≥60 years of age (Table I). The association between miR-125a-5p expression and aging requires further investigation.

Furthermore, the association between the expression level of miR-125a-5p and patient OS was assessed. Kaplan-Meier survival analysis revealed that patients with low miR-125a-5p expression exhibited a significantly shorter OS compared with those with high miR-125a-5p expression (Fig. 2D). Similarly, from the univariate analysis results of Cox proportional hazard
model analysis, the higher tumor size, advanced TNM stage, and low miR-125a-5p expression are prognostic factors for survival (Table II). While using multivariate Cox's proportional hazard model analysis, it was identified that advanced TNM stage and low miR-125a-5p expression were independent risk factors for survival (Table II). Univariate Cox analysis also indicated an association between metastasis and survival for patients with GC (Table II; P<0.001). Considering that TNM contains metastasis as a component in this staging system, it was excluded in the Cox multivariate regression analysis. Patients with low miR-125a-5p expression exhibited a 2-fold increased risk of fatal outcome compared with those with high miR-125a-5p expression. Collectively, the data suggested that miR-125a-5p may be a potential prognostic biomarker for patients with GC.

### Table I. Association between miR-125a-5p expression levels and clinical characteristics.

| Characteristic          | microRNA-125a-5p |
|-------------------------|------------------|
|                         | Low (%) | High (%) | P-value |
| Sex                     |          |          |         |
| Male                    | 55 (50.46) | 54 (49.54) | 0.855 |
| Female                  | 20 (48.72) | 21 (51.22) |         |
| Age (years)             |          |          |         |
| <60                     | 30 (38.46) | 48 (61.54) | 0.003 |
| ≥60                     | 45 (62.50) | 27 (37.50) |         |
| Differentiation         |          |          |         |
| Poor, undifferentiated  | 62 (48.82) | 65 (51.18) | 0.497 |
| Well, moderate          | 13 (56.52) | 10 (43.48) |         |
| Tumor size (cm)         |          |          |         |
| <5                      | 34 (42.50) | 46 (57.50) | 0.021 |
| ≥5                      | 43 (61.43) | 27 (38.57) |         |
| TNM stage               |          |          |         |
| I, II                   | 39 (45.88) | 46 (54.12) | 0.249 |
| III, IV                 | 36 (55.38) | 29 (44.62) |         |
| Lymph node metastasis   |          |          |         |
| Negative (N0)           | 16 (57.14) | 12 (42.86) | 0.402 |
| Positive (N1, N2, N3)   | 59 (48.36) | 63 (51.64) |         |
| Tumor embolus           |          |          |         |
| Negative                | 32 (52.46) | 29 (47.54) | 0.570 |
| Positive                | 42 (47.73) | 46 (52.27) |         |
| Metastasis              |          |          |         |
| Negative                | 50 (46.73) | 57 (53.27) | 0.206 |
| Positive                | 25 (58.14) | 18 (41.86) |         |
| Ki67                    |          |          |         |
| Negative                | 0 (0.00) | 2 (100) | 0.032 |
| <25%                    | 3 (23.08) | 10 (76.92) |         |
| 25-50%                  | 9 (40.91) | 13 (59.09) |         |
| >50%                    | 18 (66.67) | 9 (33.33) |         |

The median expression value of miR-125a-5p in cancer tissues was used as the cut-off value to determine high and low groups.

In order to determine suitable cell lines to perform functional assays to assess the effects of miR-125a-5p overexpression and inhibition, its expression was evaluated in six GC cell lines. Four GC cell lines, including NCI-N87, NUGC-3, SGC-7901 and AGS, had relatively high expression, while MGC-803 and BGC-823 had relatively low expression (Fig. 3A). Therefore, SGC-7901 cells were selected to assess the effects of miR-125a-5p silencing, and MGC-803 cells were selected to assess the effects of miR-125a-5p overexpression. Previously, we evaluated the migration and invasion ability of these gastric cancer cells by Transwell assays (20). The correlation between miR-125a-5p expression levels and migration or invasion ability was subsequently analyzed in GC cells. As indicated in Fig. 3B, the expression level of miR-125a-5p was negatively correlated with cell migration (r=-0.9583, P=0.0026) and invasion (r=-0.8865; P=0.0186) ability.

**Discussion**

The hsa-miR-125a/let-7e/miR-99b cluster is located within the 19q13.2-19q13.4 region (21). Further understanding of hsa-miR-125a/let-7e/miR-99b cluster biogenesis and function is required. In physiological conditions, high levels of all cluster members or miR-125a-5p are essential in maintaining the phenotype of mouse hematopoietic stem/progenitor cells (22). Furthermore, a single cluster member, miR-125a is sufficient to induce the amplification of hematopoietic stem cells.
suggesting that miR-125a/let-7e/miR-99b cluster members differ in their expression patterns and functions, although they are derived from a common RNA precursor (23). Increased expression of these miRNAs is essential for osteoclast differentiation from primary human monocytes (24). These studies suggest the important role of miR-125a/let-7e/miR-99b cluster members in normal cell differentiation.

The miR-125a/let-7e/miR-99b cluster or its members may be involved in human pathological processes. miR-99b/let-7e/miR-125a cluster may promote metastasis in esophageal squamous cell carcinoma (ESCC) (25). Overexpression of histiocyte-miRNA cluster was demonstrated to induce ESCC cell migration and invasion in vitro (25). The miR-125a/let-7e/miR-99b cluster members were also shown to be upregulated in synovial sarcomas, however only two cluster members, let-7e and miR-99b, promoted cancer cell proliferation (26). Similarly, two cluster members, miR-99b and miR-125a, were previously implicated in the pathogenesis of cystic fibrosis (21). Decreased expression level of miR-125a, but not of miR-99b or let-7e, was identified in hepatocellular carcinoma biopsies compared with normal liver tissues, and predicted poor prognosis of patients with hepatocellular carcinoma (27). miR-125a-5p has been reported to be a potential tumor suppressor in lung carcinoma cells (28), retinoblastoma (29) and cervical carcinoma (30). All of these studies support that these miRNAs may have diverse

Table II. Univariate and multivariate Cox regression analysis of risk factors for patients with gastric carcinoma.

| Variable                          | Univariate Cox analysis | Multivariate Cox analysis |
|-----------------------------------|-------------------------|---------------------------|
|                                  | HR (95% CI)             | P-value                   | HR (95% CI)             | P-value |
| Sex (female vs. male)            | 0.920 (0.553-1.530)     | 0.748                     | 1.286 (0.808-1.237)     | 0.288 |
| Age (≥60 vs. <60 years)          | 1.184 (0.760-1.847)     | 0.455                     | 2.550 (1.595-4.077)     | <0.001 |
| Differentiation (poor vs. well)  | 1.102 (0.607-2.000)     | 0.749                     | 2.064 (1.655-4.098)     | <0.001 |
| Ki67 (high vs. low)              | 1.502 (0.190-11.892)    | 0.610                     | 0.528 (0.338-0.825)     | 0.005 |
| Metastasis (yes vs. no)          | 3.200 (2.040-5.021)     | <0.001                    | 0.005                   | 0.003 |
| Tumor size (≥5 vs. <5 cm)        | 1.704 (1.090-2.660)     | 0.019                     | 1.286 (0.808-1.237)     | 0.288 |
| TNM stage (III and IV vs. I and II) | 2.064 (1.655-4.098)      | <0.001                  | 2.550 (1.595-4.077)     | <0.001 |
| microRNA-125a-5p (high vs. low)  | 0.528 (0.338-0.825)     | 0.005                     | 0.497 (0.314-0.789)     | 0.003 |

CI, confidence interval; HR, hazard ratio.

Figure 3. Downregulation of miR-125a-5p expression in SGC-7901 cells. (A) Analysis of miR-125a-5p expression in six gastric carcinoma cell lines. (B) Scatter graph indicating the miR-125a-5p expression, migrated and invasive cell number of each cell line. (C) miR-125a-5p expression in SGC-7901 cells transfected with inhibitors or NC. ***P<0.001 vs. NC. (D) Analysis of cell proliferation using the Cell Counting Kit-8 assay. (E) Analysis of cell migration and invasion using the Transwell assay. Scale bar, 500 mm. Data are presented as the mean ± standard deviation. Three individual experiments with at least three replicates were performed. miR, microRNA; NC, negative control; NS, not significant.
functions in disease. In accordance with the aforementioned studies, the results of the present study demonstrated that the expression of miR-125a-5p was downregulated in GC tissues compared with adjacent normal tissues.

Consistent with previous research (31-34), the results of the present study identified decreased expression of miR-125a-5p in GC tissues compared with adjacent normal tissues, and decreased miR-125a-5p expression predicted poor OS in patients with GC. From the univariate analysis results of Cox proportional hazard model analysis, higher tumor size, advanced TNM stage and low miR-125a-5p expression were associated with reduced survival of patients with GC. Furthermore, advanced TNM stage and low miR-125a-5p expression were found to be independent prognostic factors associated with survival, as assessed using multivariate Cox's analysis, which is in accordance with a previous study (30).

Decreased expression levels of miR-125a-5p were reported to be associated with enhanced malignant potential, including tumor size, tumor invasion, liver metastasis and poor prognosis (32). In the present study, the association between low expression of miR-125a-5p and tumor size was established, however miR-125a-5p was not associated with distant metastasis. Differences in sample size or distribution may contribute to the outcomes of the study. Consistent with a previous study (27), the results of the present study demonstrated that low expression level of miR-125a-5p was associated with high Ki67 expression, a cellular marker for proliferation in GC; however, evaluation of Ki67 expression in cells transfected with miR125a-5p mimics or inhibitors remains to be investigated.

However, in vitro experiments demonstrated that miR-125a-5p mimics and inhibitors had no effect on GC cell proliferation, migration and invasion. These results differ from a previous report (34), where proliferation, migration and invasion of AGS and MGC-803 cells treated with pre-miR-125a were evaluated. Since miR-125a-3p, a known tumor suppressor in GC (35), derives from the 3'-arm of the same precursor (pre-miR-125a), these inhibitory effects on GC cell proliferation, migration and invasion may be mediated by miR-125a-3p, miR-125a-5p or even both. In the present study, MGC-803 cells were transfected with miR-125a-5p mimics (specifically altering miR-125a-5p expression) and SGC-7901 cells were transfected with miR-125a-5p inhibitors. However, there were no significant changes in cell proliferation, migration and invasion. Previously, it had been reported that low expression of miR-125a-5p was able to promote the paracrine vascular endothelial growth factor A signaling pathway in GC, while the latter increased Akt phosphorylation in endothelial cells and, thereby, promoted tumor angiogenesis (36). Therefore, even though there were no changes in GC cell proliferation, migratory and invasive ability in vitro, it is possible that miR-125a-5p functions as a tumor suppressor in vivo by regulating the tumor microenvironment, including effects on tumor angiogenesis. It is still possible that miR-125a-5p may regulate...
stemness (37), a critical feature of tumor formation, just like the tumor size. Therefore, in vivo experiments assessing the effect of miR-125a-5p on tumor growth and metastasis are required. TargetScan indicates that there are 3,991 candidate target genes for miR-125a-3p; however, which ones are real targets of miR-125a-5p requires further investigation using expression correlation and dual-luciferase reporter assays.

The probe sequences of miR-125a were checked in TCGA, which were matched to the chromosome 19 plus strand from 56888319-56888404. This indicates that the miR-125a expression data obtained from TCGA includes both miR-125a-3p and miR-125a-5p expression. Only the miR-125a-5p expression was analyzed in the cohort in the present study. The expression of miR-125a-3p expression in the GC tissues should be investigated in future studies.

Taken together, the results of the present study support that reduced miR-125a-5p expression is associated with larger tumor size, high Ki67 expression and poor prognosis in patients with GC. Therefore, miR-125a-5p may have prognostic value as a tumor biomarker for patients with GC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GL performed most experiments, including the RNA extraction, quantitative PCR analysis and cell transfection. SA and JH participated in the in vitro study and acquired cell viability data. GQL designed the experiments, coordinated the project and wrote the manuscript. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Written informed consents were obtained from all patients participating in the current study and this work was approved by the Peking University Cancer Hospital and Institute Ethical Committee (approval no. 2017KT779).

Patient consent for publication

Written informed consent for publication was obtained from all patients who participated in the present study.

Competing interests

The authors declare that they have no competing interests.

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