Overexpression of serum extracellular vesicle microRNA-215-5p is associated with early tumor recurrence and poor prognosis of gastric cancer

Yunfei Zhang1,1 Fengchang Huang1,1 Ning Xu1,1 Jin Wang1,1 Dan Li2,1 Liang Yin1,1,1

1Department of Oncology, The First Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan, China. 2Department of Vascular Surgery, The First Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan, China. 3Department of Acupuncture, Yunnan Hospital of Integrated Traditional Chinese and Western Medicine, Kunming 650200, Yunnan, China.

Zhang Y, Huang F, Xu N, Wang J, Li D, Yin L. Overexpression of serum extracellular vesicle microRNA-215-5p is associated with early tumor recurrence and poor prognosis of gastric cancer. Clinics (Sao Paulo). 2021;76:e2081

*Corresponding author. E-mail: kmmu_yinliang1995@163.com

OBJECTIVES: Extracellular vesicle microRNAs (EV-miRNAs) have been demonstrated to be reliable candidate biomarkers for clinical applications. However, the clinical application potential of serum EV-miR-215-5p for gastric cancer (GC) remains poorly understood. The goal of our study was to determine the efficacy of serum EV-miR-215-5p in predicting the prognosis of GC.

METHODS: Blood samples were collected from 118 patients with GC, 60 patients with benign gastric disease and BGD and 70 healthy controls. The relative levels of serum EV-miR-215-5p were measured using quantitative real-time polymerase chain reaction (qRT-PCR).

RESULTS: Compared to patients with BGD and normal controls, GC patients exhibited remarkably higher serum EV-miR-215-5p level, especially those with early tumor recurrence (ETR). Receiver operating characteristic (ROC) curve analysis showed that serum EV-miR-215-5p was able to distinguish GC patients from BGD patients or healthy controls and GC patients with ETR from those without ETR. In addition, increased serum EV-miR-215-5p levels were notably correlated with invasive depth, TNM stage, and lymph node metastasis. Moreover, serum EV-miR-215-5p levels were greatly decreased after surgical treatment, but increased at the time of ETR. Survival analysis showed that patients with higher serum EV-miR-215-5p had shorter survival. Furthermore, serum EV-miR-215-5p was an independent risk factor for GC.

CONCLUSIONS: Serum EV-miR-215-5p might be a novel biomarker for predicting ETR and prognosis of GC.

KEYWORDS: Serum EV-miR-215-5p might be a novel biomarker for predicting ETR and prognosis of GC.

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy worldwide, and owing to its high mortality rates, it has become a global human health problem (1,2). Early diagnosis and appropriate treatment of GC are undoubtedly the best strategies for improving survival. Unfortunately, detection of GC at the initial stage is very difficult because of its asymptomatic nature. Thus, many GC patients are diagnosed at advanced stages of the disease characterized by invasion and metastasis (3,4). Accurate and efficient monitoring of the development of GC considerably contributes to ameliorating its poor prognosis (5). Early tumor recurrence (ETR) is an important predictor of unfavorable prognosis in GC. Therefore, there is a compelling need to identify robust biomarkers for predicting the ETR and prognosis of GC.

MicroRNAs (miRNAs) are a class of well-conserved non-coding RNAs (approximately 19-24 nucleotides in length) (6) that are involved in cancer development, including proliferation, invasion, metastasis, and apoptosis of cancer cells. miRNAs may function either as tumor promoters or suppressors, depending on the downstream targets they regulate (7,8). Extracellular vesicles (EVs) include exosomes and microvesicles and can be found in the blood, urine, or other bodily fluids (9). Recent studies have demonstrated that extracellular vesicle miRNAs (EV-miRNAs) can be stably detected in peripheral blood and protected against degradation by enzymes (10). Therefore, serum EV-miRNAs can potentially be employed for GC diagnosis and prognosis. For instance, serum exosomal miR-423-5p is overexpressed in GC, and its upregulation is strongly associated with lymph node metastasis (11). Similarly, serum exosomal miR-1246 levels were also markedly increased in GC (12). miR-215-5p has been previously identified as an oncomiR in GC (13-15). However, the potential clinical value of serum...
EV-miR-215-5p in GC is poorly understood. In this prospective study, we aimed to assess the expression pattern and prognostic value of serum EV-miR-215-5p in patients with GC.

**MATERIALS AND METHODS**

**Patients and samples**

This study was approved by the Ethics Committee of The First Affiliated Hospital of Kunming Medical University. Written informed consent was obtained from all the participants before serum sample collection. A total of 118 patients diagnosed with GC, 60 with benign gastric disease (BGD), and 70 healthy donors were recruited. All patients with GC underwent gastrectomy. The clinical stage was evaluated in strict accordance with the Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) 7th edition classification. None of the patients received any therapy before blood sample collection. ETR was defined as recurrence within a year after the surgery, and a total of 29 patients with GC showed ETR. Detailed clinical information of all GC patients is presented in Table 1.

Peripheral blood was collected from all participants. The samples were then centrifuged in serum-separator tubes at 3,000 x g for 10 min. The supernatant was transferred to a fresh microfuge tube and stored at -80°C until further use. Blood samples were obtained from all patients with GC two weeks after the surgical treatment and from patients with ETR at the time of ETR.

**Extracellular vesicle isolation**

EVs were extracted from serum samples using the ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer’s instructions. Briefly, followed by centrifugation at 3,000 x g for 15 min, the supernatant was mixed with 1/4 volume of ExoQuick solution. The mixture was incubated for 30 min at 4°C and then centrifuged at 1,500 x g for 30 min. EV-enriched pellets were dissolved in PBS and stored at -80°C for further analysis.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The mirVana PARIS Kit (Ambion, Austin, TX, USA) was used to extract total RNA from the EVs. RNA quality was checked on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed into complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). EV-miR-215-5p expression was analyzed using SYBR® Premix Ex Taq™ II (Takara, Dalian, China) and cDNA, and the reaction was run on a 7000 Real-Time PCR system (Applied Biosystems). Each reaction was performed in triplicate. Cel-miR-39 was used as the external control, and the relative serum EV-miR-215-5p level was calculated using the 2^(-ΔΔCt) method.

**Western blot**

The proteins were resolved on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk for 1 h at room temperature (25°C), and then probed with primary antibodies against TSG-101 (1:500 dilution, Abcam, Cambridge, UK) and CD9 (1:500 dilution; Abcam) in a cold room overnight. Subsequently, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence reagents (GE Healthcare, Piscataway, NJ, USA).

**Statistical analysis**

The Mann-Whitney U test or Kruskal-Wallis test were performed to evaluate the differences between or among the groups with respect to serum EV-miR-215-5p levels. The correlations between serum EV-miR-215-5p levels and clinicopathological variables were assessed using the Chi-square test. Receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to determine the diagnostic performance of serum EV-miR-215-5p. Overall survival (OS) and disease-free survival (DFS) were assessed using Kaplan–Meier curves and log-rank tests. Univariate and multivariate analyses were performed to identify the independent prognostic factors for GC. Significance was set at \( p \leq 0.05 \). GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA, USA) and MedCalc version 12.3.0 (MedCalc, Mariakerke, Belgium) were used for statistical analyses.

**RESULTS**

**Upregulation of serum EV-miR-215-5p in GC**

Western blotting revealed that the serum-derived EVs were positive for EV markers TSG-101 and CD9, while these were expressed at very low levels in the supernatants (Figure 1A). qRT-PCR was then used to measure the expression of serum EV-miR-215-5p in all participants. As presented in Figure 1B, serum EV-miR-215-5p levels were markedly elevated in GC patients than those in BGD patients and healthy controls \( (**p<0.001) \). Moreover, significantly higher serum extracellular vesicle miR-215-5p levels were observed in GC patients with ETR than in those without ETR \( (**p<0.001, \text{Figure 1C}) \).

| Parameters                  | Serum extracellular vesicle miR-215-5p | \( p \)   |
|-----------------------------|----------------------------------------|--------|
|                             | Low  | High |       |
| Sex                         | 0.1338                                    |
| Male                        | 70   | 39   | 31    |
| Female                      | 48   | 20   | 28    |
| Age (years)                 | 0.1648                                    |
| < 60                        | 81   | 44   | 37    |
| \( \geq 60 \)               | 37   | 15   | 22    |
| Histological type           | 0.1951                                    |
| Intestinal                  | 53   | 30   | 23    |
| Diffuse                     | 65   | 29   | 36    |
| Distant metastasis          | 0.0586                                    |
| No                          | 96   | 52   | 44    |
| Yes                         | 22   | 7    | 15    |
| Invasive depth              | 0.0032                                    |
| T1/T2                       | 58   | 37   | 21    |
| T3/T4                       | 60   | 22   | 38    |
| Lymph node metastasis       | 0.0008                                    |
| No                          | 52   | 35   | 17    |
| Yes                         | 66   | 24   | 42    |
| TNM stage                   | < 0.0001                                 |
| I/I1                        | 55   | 41   | 14    |
| II/IV                       | 63   | 18   | 45    |

Table 1 - Association between serum EV-miR-215-5p levels and clinical parameters of GC.
Diagnostic performance of serum EV-miR-215-5p in GC

As shown in Figure 2A, serum EV-miR-215-5p distinguished GC patients from healthy donors, with an AUC value of 0.866 (sensitivity=68.64%, specificity=97.14%), and the serum EV-miR-215-5p distinguished GC patients from BGD patients with an AUC of 0.808 (sensitivity=65.25%, specificity=95.00%) (Figure 2B). Importantly, serum EV-miR-215-5p exhibited an AUC of 0.908 (sensitivity=93.10%, specificity=83.15%) for distinguishing GC patients with ETR from GC patients without ETR (Figure 2C).

Correlation of serum EV-miR-215-5p with clinical variables in GC

All 118 GC patients were stratified into the high serum EV-miR-215-5p group (n=59) and low serum EV-miR-215-5p group (n=59) based on the median expression of serum EV-miR-215-5p. High serum EV-miR-215-5p levels were positively correlated with invasive depth (p=0.0032), TNM stage (p<0.0001), and lymph node metastasis (p=0.0008). However, serum EV-miR-215-5p was not correlated with sex (p=0.1338), age (p=0.1648), histological type (p=0.1951), or distant metastasis (p=0.0586).

Dynamic changes in serum EV-miR-215-5p levels after gastrectomy

Compared to pre-treatment blood samples, blood samples from patients with ETR and those without ETR exhibited significantly decreased levels of serum EV-miR-215-5p (**p<0.001) (Figure 3A-3B). In patients with ETR, serum EV-miR-215-5p levels were dramatically elevated when ETR occurred (**p<0.001) (Figure 3B).

Correlation of serum EV-miR-215-5p level with OS and DFS

The prognostic differences in terms of OS and DFS were evaluated using Kaplan-Meier survival curves. As shown in
Figure 4A, GC patients in the high serum EV-miR-215-5p group had shorter OS ($p=0.012$). Likewise, patients with higher serum EV-miR-215-5p levels tended to have a low DFS ($p=0.048$, Figure 4B).

Cox regression analysis of the factors affecting OS
Univariate analysis demonstrated that invasive depth (HR=2.65, 95% CI=1.12-4.35, $p=0.036$), lymph node metastasis (HR=3.12, 95% CI=1.48-6.09, $p=0.008$), TNM stage (HR=3.98, 95% CI=1.87-8.97, $p<0.001$), and serum EV-miR-215-5p levels (HR=2.95, 95%CI=1.31-5.68, $p=0.013$) were significantly associated with OS in GC. Multivariate analysis revealed that lymph node metastasis (HR=2.71, 95% CI=1.36-5.32, $p=0.012$), TNM stage (HR=3.67, 95%CI=1.61-7.58, $p=0.003$), and serum EV-miR-215-5p levels (HR=2.28, 95% CI=1.23-4.36, $p=0.028$) were independent prognostic factors for GC (Table 2).

### DISCUSSION
To our knowledge, this was the first study to evaluate the clinical significance of serum EV-miR-215-5p levels in GC. First, we found that serum EV-miR-215-5p levels were elevated in GC patients, especially in patients with ETR. Second, ROC analysis revealed that serum EV-miR-215-5p was a promising biomarker for distinguishing GC patients with ETR from those without ETR. Third, a strong association was found between high serum EV-miR-215-5p levels and unfavorable clinical outcomes. Fourth, the serum EV-miR-215-5p level was sensitive to therapeutic responses. Finally, the serum EV-miR-215-5p was an independent prognostic factor for GC. In conclusion, serum extracellular vesicle miR-215-5p may serve as a non-invasive biomarker for predicting the prognosis of GC and monitoring therapeutic responses.
Consistent with our results, miR-215-5p expression is significantly upregulated in GC tissues. Overexpression of miR-215-5p greatly stimulates the migration and invasion of cancer cells by resulting in the degradation of FOXO1 (13). Similarly, upregulation of miR-215-5p is observed in GC tissues and cell lines, which results in significantly enhanced carcinogenesis (14). miR-215-5p expression is significantly increased in GC tissues and cell lines than that in their respective controls. Ectopic expression of miR-215-5p dramatically promotes the malignancy of GC cells, and vice versa (15,16). miR-215-5p upregulation occurs more frequently in high-grade glioma, and miR-215-5p overexpression has been positively correlated with poor prognosis. In addition, in vitro analysis showed that enforced miR-215-5p expression promotes migration and invasion of glioma cells by targeting RB1, and vice versa (17,18).

In contrast, miR-215-5p may also play a tumor-suppressive role in various cancer types. miR-215-5p expression is significantly lower in non-small cell lung cancer (NSCLC) cell lines and tissues. Upregulation of miR-215-5p suppressed the proliferation, migration, and apoptosis of NSCLC cells in vitro, and inhibited tumorigenesis in vivo (19-21). miR-215-5p levels are decreased in acute myeloid leukemia, and its downregulation is associated with shorter survival (22). Similarly, miR-215-5p is underexpressed in papillary thyroid cancer (PTC) tissues and cell lines. Enforced miR-215-5p expression dramatically limits the tumorigenicity of cancer cells by regulating the expression of ARFGEF1 (23). MiR-215-5p is frequently downregulated in epithelial ovarian cancer (EOC) tissues and cell lines. Overexpression of miR-215-5p significantly suppresses EOC progression both in vitro and in vivo (24,25).

In summary, serum EV-miR-215-5p levels are elevated in GC, and their upregulation is strongly associated with poor prognosis in GC. Therefore, serum EV-miR-215-5p may serve as a novel and robust biomarker for stratifying GC patients with different outcome risks.

**AUTHOR CONTRIBUTIONS**

Zhang Y and Yin L designed the study and supervised the experiments. Zhang Y, Huang F, Xu N, Wang J, Li D, and Yin L collected the data, performed the experiments, analyzed the data, and wrote the manuscript.

**REFERENCES**

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87-108. https://doi.org/10.3322/caac.21262
2. Chen W, Zheng R, Baade PD, Zong S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. CA Cancer J Clin. 2016;66(2):115-32. https://doi.org/10.3322/caac.21338
3. Jou E, Rajdev L. Current and emerging therapies in unresectable and recurrent gastric cancer. World J Gastroenterol. 2016;22(20):4812-23. https://doi.org/10.3748/wjg.v22.i20.4812
4. Yan JY, Tian FM, Hu WN, Zhang JH, Cai HF, Li N. Apoptosis of human gastric cancer cells line SGC 7901 induced by garlic-derived compound S-allylmercaptocysteine (SAMC). Eur Rev Med Pharmacol Sci. 2013;17(6):745-51.
5. Landsorp-Vogelaar I, Kuipers EJ. Screening for gastric cancer in Western countries. Gut. 2016;65(3):434-4. https://doi.org/10.1136/gutjnl-2013-305679
6. Shukla GC, Singh J, Barik S. MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. Mol Cell Pharmacol. 2011;5(3):83-92.
7. Zhao X, Cui L. A robust six-miRNA prognostic signature for head and neck squamous cell carcinoma. J Cell Physiol. 2020;235(11):8799-811. https://doi.org/10.1002/jcp.29722
8. Wang DZ, Zhang B, Zhang MS, Gao Y. Upregulation of serum miR-103 predicts unfavorable prognosis in patients with colorectal cancer. Eur Rev Med Pharmacol Sci. 2018;22(14):4518-23.
9. Kowal J, Tkach M, Thery C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol. 2014;29:116-25. https://doi.org/10.1016/j.jceb.2014.05.004
10. Valadi H, Ekstrom K, Bossaies C, Sjostrand M, Lee J, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654-9. https://doi.org/10.1038/ncb1596
11. Yang H, Fu H, Wang B, Zhang X, Mao J, Li X, et al. Exosomal miR-423-5p targets SULU to promote cancer growth and metastasis and serves as a novel marker for gastric cancer. Mol Carcinog. 2018;57(9):1223-36. https://doi.org/10.1002/mc.22838
12. Shi Y, Wang Z, Zha X, Chen L, Ma Y, Wang J, et al. Exosomal miR-1246 in serum as a potential biomarker for early diagnosis of gastric cancer. Int J Clin Oncol. 2020;25(1):89-99. https://doi.org/10.1007/s10147-019-01532-9
13. Zhang Y, Wang T, Pan J, Gao F. miR-215 promotes cell migration and invasion of gastric cancer cell lines by targeting FOXO1. Pathol Res Pract. 2017;213(8):889-94. https://doi.org/10.1016/j.prp.2017.06.006
14. Li N, Zhang QY, Zou JL, Tian ZZ, Dong B, et al. Exosomal miR-215 promotes malignant progression of gastric cancer by targeting RUNX1. Oncotarget. 2017;8(46):93387-93401. https://doi.org/10.18632/oncotarget.2017.83832
15. Wei Y, Sun J, Li X. MiRNA-miRNA pairs enhance invasion and migration by targeting retinoblastoma tumor suppressor gene 1. Pathol Res Pract. 2017;213(4):2349-53. https://doi.org/10.1016/j.prp.2017.03.033
16. Wei Y, Sun J, Li X. MiRNA-miRNA pairs enhance invasion and migration by targeting retinoblastoma tumor suppressor gene 1. Pathol Res Pract. 2017;213(4):2349-53. https://doi.org/10.1016/j.prp.2017.03.033
17. Wei Y, Sun J, Li X. MicroRNA-215 enhances invasion and migration by targeting retinoblastoma tumor suppressor gene 1 in high-grade glioma. Biotechnol Lett. 2017;39(2):197-205. https://doi.org/10.1007/s10529-016-2251-8
18. Meng X, Shi B. miR-215 functions as an oncogene in high-grade glioma by regulating retinoblastoma 1. Biotechnol Lett. 2017;39(9):1351-8. https://doi.org/10.1007/s10529-017-2373-7
19. Cai X, Peng D, Wei H, Yang X, Huang Q, Lin Z, et al. MiR-215 suppresses proliferation and migration of non-small cell lung cancer cells. Oncol Lett. 2017;13(4):2349-53. https://doi.org/10.3892/ol.2017.5692
20. Hou Y, Zhen J, Xu X, Zhen K, Zhu B, Pan R, et al. miR-215 functions as a tumor suppressor and directly targets ZEB2 in human nonsmall cell lung cancer. Oncol Lett. 2015;10(4):1985-92. https://doi.org/10.3892/ol.2015.3587
21. Yao Y, Shen H, Zhou Y, Yang Z, Hu T. MicroRNA-215 suppresses the proliferation, migration and invasion of non-small cell lung carcinoma cells through the downregulation of matrix metalloproteinase-16 expression. Exp Ther Med. 2018;15(4):3239-46.
22. Wang YX, Zhang TJ, Yang DQ, Yao DM, Yang L, Zhou JD, et al. Reduced miR-215 expression predicts poor prognosis in patients with acute myeloid leukemia. Jpn J Clin Oncol. 2016;46(4):350-6. https://doi.org/10.1093/jjco/hyv204

23. Han J, Zhang M, Nie C, Jia J, Wang F, Yu J, et al. miR-215 suppresses papillary thyroid cancer proliferation, migration, and invasion through the AKT/GSK-3β/Snail signaling by targeting ARFGEF1. Cell Death Dis. 2019;10(3):195. https://doi.org/10.1038/s41419-019-1444-1

24. Lin Y, Jin Y, Xu T, Zhou S, Cui M. MicroRNA-215 targets NOB1 and inhibits growth and invasion of epithelial ovarian cancer. Am J Transl Res. 2017;9(2):466-77.

25. Ge G, Zhang W, Niu L, Yan Y, Ren Y, Zou Y. miR-215 functions as a tumor suppressor in epithelial ovarian cancer through regulation of the X-chromosome-linked inhibitor of apoptosis. Oncol Rep. 2016;35(3):1816-22. https://doi.org/10.3892/or.2015.4482