Original Paper

MiR-520b/e Regulates Proliferation and Migration by Simultaneously Targeting EGFR in Gastric Cancer

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Key Words

miR-520b/e • EGFR • Gastric cancer • Proliferation • Migration

Abstract

Background: MicroRNAs (miRNAs) have been demonstrated to play a crucial role in tumorigenesis. Previous studies have shown that miR-520b/e acts as a tumor suppressor in several tumors. Other studies indicated that epidermal growth factor receptor (EGFR) is highly expressed in many tumors, and involved in the development of tumors, such as cell proliferation, migration, angiogenesis and apoptosis. However, the correlation of miRNAs and EGFR in gastric cancer (GC) has not been adequately investigated. Our aim was to explore the relationship. **Methods:** The expression levels of EGFR and miR-520b/e were examined by RT-PCR and Western blot. We also investigated the relationship between EGFR and miR-520b/e in GC cell lines by relevant experiments. **Results:** In this study, we found that miR-520b/e inhibits the protein expression of EGFR by directly binding with the 3'-untranslated region (3'-UTR). And it was shown that the down-regulation of miR-520b/e promotes cell proliferation and migration by negative regulation of the EGFR pathway, while over-expression of miR-520b/e inhibits these properties. In addition, the biological function of EGFR in GC cell lines was validated by silencing and over-expression assays respectively. **Conclusions:** Taken together, our results demonstrate that miR-520b/e acts as a tumor suppressor by regulating EGFR in GC, and provide a novel marker and insight for the potential therapeutic target of GC.

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Introduction

Gastric cancer (GC) is the second-most common malignant tumor in the world, and the survival rate is various in different countries [1, 2]. The prognosis of GC is closely related to the stage, grade and metastasis [3]. The main treatment for GC are surgery, radiotherapy and chemotherapy, but the effect is limited [4]. Targeted therapy provides a new way for

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the treatment of GC, and can reduce unnecessary side effects [5]. Therefore, exploring the molecular mechanism of GC is very useful for the diagnosis and therapy of this disease.

Epidermal growth factor receptor (EGFR) is a member of HER family, which is widely distributed in the surface of mammalian cell membranes [6]. EGFR, also known as HER1 or ErbB1, plays an important role in cell migration, proliferation, cell cycle regulation and other physiological processes [7]. Previous studies have indicated that the aberrant expression of EGFR is associated with development and prognosis of a number of solid tumors [8, 9]. However, the underlying mechanism of EGFR in GC proliferation and migration remains elusive and needs to be further investigated.

MicroRNAs (miRNAs) are noncoding RNAs of 21-25 nucleotide-long that regulate protein translation through imperfect complementarity with the 3'-untranslated region (3'-UTR) of target messenger RNA (mRNA) [10]. Most miRNAs are highly conservative and tissue specificity that act as post-transcriptional regulators [11]. In normal cells, miRNAs play an important role in regulating cell growth and differentiation [12]. Accumulating evidences have been demonstrated that miRNAs can function as oncogenes or tumor suppressors in cancer progression, including tumor proliferation and migration [13, 14]. Previous studies have shown that miR-520b/e is down-expressed in many tumors, and serves as a tumor suppressor [15, 16]. However, as far as we known, the function of miR-520b/e in the growth of GC remains unclear.

In the present study, we illustrated the connection between miR-520b/e and EGFR in GC. Luciferase assays confirmed that miR-520b/e is directly associated with the 3'UTR of the mRNA of EGFR. Our data suggested that miR-520b/e acts as a tumor suppressor by negatively regulating EGFR expression. The expression of EGFR was increased or decreased by transfecting of miR-520b/e mimics and inhibitors, respectively. Subsequently, functional experiments confirmed that miR-520b/e inhibits the occurrence and development of GC.

Therefore, our findings indicate that the miR-520b/e acts as EGFR inhibitor, and provides a new target for the treatment of GC.

Materials and Methods

Human tissue

Gastric cancer tissues and their paired adjacent noncancerous tissues were derived from patients undergoing a radical surgery at the Tianjin Medical University Cancer Institute and Hospital. Both tumor tissues were histopathologically verified adenocarcinoma and noncancerous tissues were confirmed negative. Tissue fragments were immediately frozen in liquid nitrogen at the time of surgery. Total protein and RNA of those paired tissues were extracted and stored at -80 °C.

Patients and ethics statement

The study was approved by the the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and conformed to the standards set by the Declaration of Helsinki. All participants provided written informed consent to participate in the study.

Cell culture

Human gastric cell line SGC-7901 and MGC-803 were cultured in DMEM (Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Solarbio, China) in a humidified incubator at 37 °C with 5% CO₂.

The miRNA target prediction and Luciferase reporter assay

The miRNA target prediction and analysis were performed with the algorithms from TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>) and miRanda (<http://www.microrna.org/>). The reporter plasmid p-MIR-EGFR containing the predicted miR-520b/e targeting regions was designed by Genescript (Nanjing, China). Part of the wild type and mutated 3'UTR of EGFR was cloned immediately downstream of the firefly luciferase reporter. The 2 mg of β -galactosidase expression vector

(Ambion) was used as a transfection control. For the subsequent luciferase reporter assays, 2 mg of firefly luciferase reporter plasmid, 2 mg of β -galactosidase vector, and equal doses (200 pmol) of mimics, inhibitors, or scrambled negative control RNA were transfected into the prepared cells. At 24 h after transfection, cells were analyzed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Each sample was prepared in triplicate, and the entire experiment was repeated three times.

Cell transfection

SGC-7901 and MGC-803 cells were plated in six-well or other plates and performed transfection after 24 h. The EGFR overexpressing plasmid (OEDNA EGFR) and the control plasmid (OEDNA NC) were bought from GenePharma (Shanghai, China); and 2g of plasmid were transfected into every single well. Cells were transfected with scrambled negative control, miR-520b/e mimics or inhibitors using lipofectamine 2000 (Invitrogen, Life Technologies) and Opti-MEM Reduced Serum Medium (Gibco, Life Technologies) according to the manufacturer's instructions. And equal doses (100 pmol) of miRNA mimics, inhibitors, siRNAs (Santa Cruz), or scrambled negative control RNA were transfected into each well. Then the cells were harvested at 24 h after transfection for real-time quantitative PCR analysis and at 48h for western blotting.

RT-PCR

Total RNA was extracted from the cultured cells and tissues using TRIzol Reagent (Invitrogen, USA) following as the manufacturer's protocol. MiRNA was quantitated using Taqman microRNA probes (Applied Biosystems, Foster City, CA). After the reactions were completed, the cycle threshold (C_t) data were determined using fixed threshold settings, and the mean C_t was calculated from triplicate PCRs. A comparative C_t method was used to compare each condition to the control reactions. U6 snRNA was used as an internal control of miRNAs, and the EGFR mRNA levels were normalized to the corresponding housekeeping gene GAPDH. The relative amount of gene normalized to control was calculated with the equation $2^{-\Delta C_t}$, in which $\Delta C_t = C_{t \text{ gene}} - C_{t \text{ control}}$. All of the reactions were performed in triplicate. Primers of EGFR and GAPDH were designed as follows:

5'-AGAAGGCTGGGGCTCATTTG-3' (GAPDH, sense);

5'-AGGGGCCATCCACAGTCTTC-3' (GAPDH, antisense);

5'-GAGCCTCTGGATGGTGCAAT-3' (EGFR, sense);

5'-GAGCCTCTGGATGGTGCAAT-3' (EGFR, antisense).

Western blotting

The EGFR expression was assessed by western blotting analysis and samples were normalized to GAPDH. Total proteins were extracted from the cultured cells were solubilized in lysis buffer. The protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Roche). The membranes were blocked within 2% Bovine Serum Albumin (BSA) at room temperature for 1 h and incubated overnight at 4 °C with primary anti-EGFR (1:2000, Santa Cruz) and anti-GAPDH (1:5000, Santa Cruz), respectively. The membranes subsequently washed and incubated with appropriate secondary antibodies. After incubated with ECL, the protein bands were visualized.

Cell proliferation assay

The proliferative ability of SGC-7901 and MGC-803 cells after different transfection was determined by the EdU proliferation assay (RiboBio Inc.). Twenty-four hours after transfection, cells were incubated with 50 μ M EdU for 5 h, and fixed within 4% paraformaldehyde for 30 min at room temperature (RT). Then the cells were washed in PBS twice and permeabilized using PBS containing 0.5% Triton X-100 for 10 min. After extensive washes in PBS, the cells were incubated lucifugally in Apollo staining solution (RiboBio Inc.) for 30 min, then repeated permeation and wash, and incubated in Hoechst 33342 (1:100; RiboBio Inc.) for another 30 min at RT. All of the staining were performed in triplicate.

Cell migration assay

The migration ability of SGC-7901 and MGC-803 cells after different transfection was measured by wound healing assay and transwell assay. When the cells were about 90%, a wound healing assay was performed. After washing with PBS, medium with 1% FBS was added. We observed and photographed at

0 hour, 6 hours, 18 hours and 24 hours after scraping. The distance of the wound zone in each well was measured at least three randomly selected fields and compared with control. Then, approximately 10^5 cells were seeded on the upper chamber of the transwell with 200 μ l serum-free growth medium (10^5 cells per well of 8.0 μ m Pore Polycarbonate Membrane Insert). Complete medium containing 10% FBS was added to the lower chamber as a chemo-attractant. After 24h of incubation at 37 °C, non-migratory cells on the upper surface of upper chamber were removed slightly by cotton swabs, and cells that migrated to the bottom of the membrane were fixed and stained. The number of invaded cells was counted under light microscope. To minimize the bias, five randomly selected fields with 200 $\times$ magnification were counted, then the average number was calculated.

Immunohistochemistry assays

Formalin-fixed, paraffin-embedded sections of tissue specimens including gastric adenocarcinoma and paired adjacent noncancerous tissues were reviewed by pathologists. EGFR-examinations in tissues were performed on 8- μ m-thick paraffin sections. All sections were deparaffinized twice with xylene and rehydrated in a graded series of ethanol. The sections were soaked in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval, and heated to 220°C in high pressure for 3 minutes. Endogenous peroxidase activity was blocked by soaking in 3% hydrogen peroxide for 20 minutes. The sections were then incubated overnight at 4°C with anti-human EGFR monoclonal antibody (1:50, Santa Cruz, sc-400). The next day, the slides were washed in PBS and incubated with second antibodies for 40 minutes at 37 °C. After washes with PBS, 3-amino-9-ethylcarbazole solution were used to chromogen. Then the sections were counterstained with hematoxylin, dehydrated, and coverslipped. Quantitative analysis was conducted by quantifying the fluorescence intensity from six sections.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics, Version 20.0. All data were representative of at least three independent experiments. Data were described with median values $\pm$ SME and analyzed by using the Student's t test.

Differences were considered statistically significant for $P < 0.05$. In this study, '*' indicates ' $P < 0.05$ ', '**' indicates ' $P < 0.01$ ', and '***' indicates ' $P < 0.001$ '

Results

EGFR is up-regulated in GC tissues

To determine the expression of EGFR in three GC patients, we collected the GC tissues and adjacent noncancerous tissues. The expression of EGFR in GC tissues was detected by western blotting assay. Compared with the normal adjacent noncancerous tissues, the protein levels of EGFR were dramatically up-regulated in GC tissues (Fig. 1A). However, there was slight increase in GC tissues compared to noncancerous tissues about the mRNA levels of EGFR (Fig. 1C). IHC assays confirmed that the expression characteristic of EGFR is positive cytoplasm staining, which expresses GC tissues instead of normal adjacent tissues (Fig. 1B). This indicated that the expression of EGFR is regulated at the post-transcriptional levels.

MiR-520b/e is down-regulated in the GC tissues

The expression level of miR-520b/e in GC tissues and paired adjacent noncancerous tissues was evaluated by real-time PCR in this study. Compared with the adjacent noncancerous tissues, significant decreased of miR-520b/e was observed in GC tissues (Fig. 1E). Meanwhile, we used bioinformatics tools to elucidate whether EGFR is a potential downstream target of miR-520b/e. It was found that miR-520b/e directly binds to the 3'UTR of EGFR mRNA. The binding sites were shown in the Figure 1D and Figure 2A. This result implied that miR-520b/e is one of the most important regulators of EGFR in GC cells.

MiR-520b/e negatively regulates EGFR expression

To validate whether EGFR is a direct functional target of miR-520b/e, luciferase assays were performed. The relative luciferase activity was detected when miR-520b/e mimics or

Fig. 1. Inverse correlation between EGFR and miR-520b/e in GC tissues. A. Western blot analysis of EGFR expression in GC cancer tissues and the paired adjacent noncancerous tissues (n=3). B. Immunohistochemistry assays of EGFR expression in GC cancer tissues and adjacent noncancerous tissues (n=3). C. Relative levels of EGFR mRNA in GC tissues and normal tissues (n=3). D. The predicted binding sites of miR-520b/e in the mRNA of EGFR (n=3). E. Relative levels of miR-520b/e in GC tissues and normal tissues. NC is the paired non-cancerous group of GC. ** indicates $p < 0.01$.

Fig. 2. The luciferase report gene analysis miR-520b/e. A. Schematic description of the base-pairing interaction between miR-520b/e and EGFR mRNA. B, C and D. Direct recognition of EGFR by miR-520b/e. GC cells were co-transfected with firefly luciferase reporters containing either WT or mutant EGFR 3'UTR with miR-520b/e mimics and inhibitors. An interaction between miR-520b/e and the target was evident (n=3). NC is the corresponding negative control of mimics or inhibitors. ** indicates $p < 0.01$.

inhibitors and their corresponding NCs were co-transfected with the luciferase reporters containing the predicted wild or mutated binding sites in the 3'UTR of EGFR. The plasmid containing the wild EGFR 3'UTR was indicated that co-transfection of miR-520b/e mimics

Fig. 3. MiR-520b/e regulates EGFR expression in SGC-7901 cells. **A.** Quantitative RT-PCR analysis of the relative miR-520b/e levels of SGC-7901 treated with miR-520b/e mimics and inhibitors (G) (n=3). **B.** The suppression of EGFR expression and quantitative analysis by miR-520b/e in SGC-7901 cells (n=3). **C.** Quantitative RT-PCR analysis of EGFR mRNA levels in SGC-7901 cells treated with miR-520b/e mimics and inhibitors (n=3). NC is the corresponding negative control of mimics or inhibitors. ** indicates p<0.01.

Fig. 4. MiR-520b/e regulates EGFR expression in MGC-803 cells. **A.** The suppression of EGFR expression by miR-520b/e in MGC-803 cells (n=3). **B** and **C.** Quantitative analysis of **A** (n=3). NC is the corresponding negative control of mimics or inhibitors. ** indicates p<0.01.

significantly decreased luciferase signal, while the co-transfection of miR-520b/e inhibitors increased luciferase signal. However, the interaction was lost when a plasmid with a mutated sequence was used instead (Fig. 2B, 2C and 2D).

Fig. 5. MiR-520b/e regulates the proliferation and migration of SGC-7901 cells. A. EdU assays demonstrate that miR-520b/e suppress the proliferation of SGC-7901 cells. Overexpression of miR-520b/e suppress cell proliferation, whereas low levels of miR-520b/e promote SGC-7901 cell proliferation (n=3). B. Quantitative analysis of A (n=3). C. Transwell assays demonstrate that miR-520b/e suppress the migration of SGC-7901 cells. Overexpression of miR-520b/e suppress cell migration, whereas low levels of miR-520b/e promote SGC-7901 cell migration in GC (n=3). D. Quantification of C (n=3). E. Validation of

miR-520b/e-mediated cell migration by wound healing method (n=3). NC is the corresponding negative control of mimics or inhibitors. ** indicates $p < 0.01$.

In order to clarify the function of miR-520b/e, miR-520b/e mimics or inhibitors and their corresponding NCs were transfected into GC cells. After 24h, the cells were collected for detecting miR-520b/e levels by qRT-PCR analysis. Results showed that compared with the control group, miR-520b/e mimics could significantly increase miR-520b/e level in GC cells, while miR-520b/e inhibitors decreased miR-520b/e level (Fig. 3A). Then, the effect of miR-520b/e on expression of EGFR was examined by western blot in SGC-7901 cells. MiR-520b/e mimics led to reduction of EGFR, whereas miR-520b/e inhibitors enhanced the expression of EGFR (Fig. 3B). Furthermore, miR-520b/e did not affect the mRNA levels of EGFR in GC (Fig. 3C).

MiR-520b/e mimics or inhibitors and their corresponding NCs were also transfected into MGC-803 cells. MiR-520b/e mimics led to reduction of EGFR, whereas miR-520b/e inhibitors enhanced the expression of EGFR by western blot (Fig. 4A, 4B and 4C).

Fig. 6. MiR-520b/e regulates the proliferation and migration of MGC-803 cells. **A.** EdU assays demonstrate that miR-520b/e suppress the proliferation of MGC-803 cells. Overexpression of miR-520b/e suppress cell proliferation, whereas low levels of miR-520b/e promote MGC-803 cell proliferation (n=3). **B.** Quantitative analysis of A (n=3). **C.** Transwell assays demonstrate that miR-520b/e suppress the migration of MGC-803 cells. Overexpression of miR-520b/e suppress cell migration, whereas low levels of miR-520b/e promote MGC-803 cell migration in GC (n=3). **D.** Quantification of C (n=3). **E.** Validation of miR-520b/e-mediated cell migration by wound healing method (n=3). NC is the corresponding negative control of mimics or inhibitors. ** indicates p<0.01.

In conclusion, miR-520b/e is an important post-transcriptional molecular by regulating 3'UTR of EGFR in GC cells.

MiR-520b/e inhibits proliferation and migration of SGC-7901 and MGC-803 cells

We next examined the biological effects of miR-520b/e and EGFR on GC cells. We transfected miR-520b/e mimics, inhibitors or miRNA NCs in GC cell line. The 5-ethynyl-2-

Fig. 7. Overexpressing or silencing EGFR influences EGFR protein levels in GC cells. A and B. Western blot used in overexpressing or silencing of EGFR expression by overexpression plasmid or siRNA. GC cells were transfected with plasmid or siRNA, and the protein levels were measured (n=3). C. and D. The mRNA levels of A were measured (n=3). OE EGFR refers to overexpression plasmid. OE.NC is the negative control of EGFR overexpression plasmid. Si.NC is the negative control of siRNA of EGFR. ** indicates $p < 0.01$.

deoxyuridine (EdU) proliferation incorporation assay was used to measure the proliferation of SGC-7901 cells. We found that cells transfected miR-520b/e mimics were decreased, whereas cells transfected miR-520b/e inhibitors showed increase compared with the NC group (Fig. 5A and 5B).

We also conducted transwell assay (Fig. 5C and 5D) and wound healing method (Fig. 5E) to evaluate the migration capacity of SGC-7901 cells. Compared with the control group, the overexpression of miR-520b/e inhibited the migration of GC cells, while the transfection of miR-520b/e promoted cell migration.

We also demonstrated the role of miR-520b/e in promoting the proliferation and migration in MGC-803 cells. We can see that miR-520b/e mimics decreased the proliferation of MGC-803, whereas miR-520b/e inhibitors increased the trend by EdU assay (Fig. 6A and 6B). Similar results also occurred in migration of MGC-803 by transwell and wound healing assay (Fig. 6C, 6D and 6E).

These results demonstrated that miR-520b/e acts as a tumor suppressive miRNA in human GC that could weaken proliferation and migration ability in SGC-7901 and MGC-803 cells.

Effects of EGFR on proliferation and migration in GC cells

To further explore the role of EGFR in cell proliferation and migration, we used plasmid or siRNA to realize the overexpression or silence of EGFR. The siRNA sequence targeting human EGFR cDNA was used to knock-down the expression of EGFR, and a plasmid expressing the ORF of EGFR was designed to overexpress EGFR. The silencing and overexpressing efficiency was assessed by RT-qPCR for RNA and by western blotting for protein. As is shown in the figures, both the mRNA and protein levels of EGFR were markedly inhibited by siRNA or augmented by the overexpression plasmid respectively, compared with control (Fig. 7). In addition, we used EdU proliferation assay, wound healing and transwell assay to testing GC cells in which EGFR was knocked-down exhibited a significantly lower rate of proliferation and decreased migration, whereas the cells overexpressing EGFR exhibited a significantly higher rate of proliferation and increased migration (Fig. 8). Therefore, EGFR may act as a cancer accelerant in GC and increase the rate of proliferation and the ability of migration in cancer cells.

Fig. 8. Overexpressing or silencing of EGFR regulates cell proliferation and migration in GC cells. A. Edu assays demonstrate that overexpression of EGFR promotes cell proliferation of GC cells, whereas silencing of EGFR inhibits cell proliferation (n=3). B. Quantitative analysis of A (n=3). C. Transwell assays show that overexpression of EGFR enhances cell migration, whereas silencing of EGFR inhibits cell migration (n=3). D. Quantitative analysis of C (n=3). E. Validation of EGFR-mediated cell migration by wound healing method. OE EGFR refers to overexpression plasmid. OE.NC is the negative control of EGFR overexpression plasmid. Si.NC is the negative control of siRNA of EGFR. ** indicates $p < 0.01$.

Discussion

With the progress of diagnostic methods and the improvement of new treatments, the survival rate of many tumors is increasing [17]. But as a kind of common malignant tumor, the mortality rate of GC is still high, especially in the middle and late stage [18]. Because

GC is prone to local invasion and lymph node metastasis, radiotherapy and chemotherapy have limited effect on survival [19]. The molecular targeted therapy provides a new insight for the treatment of GC, but the technology is not mature [20]. So, we need to identify high specificity miRNAs and their targets.

Accumulating data indicated that miRNAs are associated with cancer development, including migration and proliferation [14, 21]. MiRNAs affect tumorigenesis by regulating downstream genes at the post-transcriptional level [11, 22]. Because of the stability of miRNA, many

studies regard it as a biomarker in human cancers, by acting as oncogenes or suppressors [23, 24]. In various types of cancers, miRNA-related pathways is a heat-point, such as miR-17 and miR-29a/c in gastric cancer [25, 26], miR-135b in colorectal cancer [27], miR-106a in cholangiocarcinoma [28]. MiRNAs are also related to development and progression of GC [29].

In previous studies, miR-520 have reported a suppressor in tumors, by regulating CD46 in breast cancer [15], NIK in hepatocellular carcinoma [16], CD44 in prostate cancer [30], MMP9 in fibrosarcoma [31], CRCT1 in esophageal squamous cell cancer [32], CTHRC1 in colorectal cancer [33]. MiR-520 also has been reported in GC [34]. However, to our knowledge, the study of the relationship between miR-520b/e and EGFR pathway in GC has not been reported. Our study confirms that miR-520b/e acts as a tumor suppressor gene in GC.

In the current study, we found an increase expression of EGFR but a decrease of miR-520b/e in GC tissues compared with adjacent noncancerous tissues. The protein expression of EGFR was negatively correlated with miR-520b/e, but the amount of mRNA was little changed at the same time. We raised or lowered miR-520b/e by transfection of miR-520b/e mimics or inhibitors. In SGC-7901 and MGC-803 cells, miR-520b/e mimics inhibit the expression of EGFR protein, but miR-520b/e inhibitors promote protein expression. The next functional experiment confirmed that up-regulation of miR-520b/e inhibition and down-regulation of miR-520b/e to promote tumor proliferation and migration by EGFR. After the relevant experiments, through the interference of plasmids or over expression of the virus to down or up EGFR expression, to determine the EGFR in cancer tumor promoting function. So these data indicate that miR-520b/e can regulate the proliferation and migration of tumor cells through the target EGFR. Of course, miR-520b/e-mediated molecular pathway in GC still needs further exploration.

In summary, our data revealed that miR-520b/e regulation of EGFR is a key factor in the development of GC cells, which provides a potential target for clinical treatment of GC patients.

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Disclosure Statement

The authors declare that there is no conflict of interests regarding the publication of this article.

References

- 1 Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, Bannon F, Ahn JV, Johnson CJ, Bonaventure A, Marcos-Gragera R, Stiller C, Azevedo e Silva G, Chen WQ, Ogunbiyi OJ, Rachet B, Soeberg MJ, You H, Matsuda T, Bielska-Lasota M, Storm H, Tucker TC, Coleman MP: Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* 2015;385:977-1010.
- 2 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 2015;65:87-108.
- 3 Kim JP, Lee JH, Kim SJ, Yu HJ, Yang HK: Clinicopathologic characteristics and prognostic factors in 10 783 patients with gastric cancer. *Gastric Cancer* 1998;1:125-133.
- 4 Liakakos T, Xeropotamos N, Ziogas D, Roukos D: EGFR as a Prognostic Marker for Gastric Cancer. *World J Surg* 2008;32:1225-1226; author reply 1227-1229.
- 5 Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H: Gastric cancer. *Lancet* DOI:10.1016/s0140-6736(16)30354-3.
- 6 Bazley LA, Gullick WJ: The epidermal growth factor receptor family. *Endocr Relat Cancer* 2005;12:S17-27.
- 7 Tamas P, Solti Z, Bauer P, Illes A, Sipeki S, Bauer A, Farago A, Downward J, Buday L: Mechanism of epidermal growth factor regulation of Vav2, a guanine nucleotide exchange factor for Rac. *J Biol Chem* 2003;278:5163-5171.
- 8 Rocha-Lima CM, Soares HP, Raez LE, Singal R: EGFR targeting of solid tumors. *Cancer Control* 2007;14:295-304.
- 9 Nicholson RI, Gee JM, Harper ME: EGFR and cancer prognosis. *Eur J Cancer* 2001;37:S9-15.
- 10 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
- 11 Liu X, Fortin K, Mourelatos Z: MicroRNAs: biogenesis and molecular functions. *Brain Pathol* 2008;18:113-121.
- 12 Ambros V: The functions of animal microRNAs. *Nature* 2004;431:350-355.
- 13 Chen CZ: MicroRNAs as oncogenes and tumor suppressors. *N Engl J Med* 2005;353:1768-1771.
- 14 Esquela-Kerscher A, Slack FJ: Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 2006;6:259-269.
- 15 Cui W, Zhang Y, Hu N, Shan C, Zhang S, Zhang W, Zhang X, Ye L: miRNA-520b and miR-520e sensitize breast cancer cells to complement attack via directly targeting 3'UTR of CD46. *Cancer Biol Ther* 2010;10:232-241.
- 16 Ambros V: MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* 2003;113:673-676.
- 17 Valverde CM, Macarulla T, Casado E, Ramos FJ, Martinelli E, Tabernero J: Novel targets in gastric and esophageal cancer. *Crit Rev Oncol Hematol* 2006;59:128-138.
- 18 Bilici A: Treatment options in patients with metastatic gastric cancer: current status and future perspectives. *World J Gastroenterol* 2014;20:3905-3915.
- 19 Cunningham D, Chua YJ: East meets west in the treatment of gastric cancer. *N Engl J Med* 2007;357:1863-1865.
- 20 Okines AF, Dewdney A, Chau I, Rao S, Cunningham D: Trastuzumab for gastric cancer treatment. *Lancet* 2010;376:1736; author reply 1736-1737.
- 21 Calin GA, Croce CM: MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-866.
- 22 Stahlhut Espinosa CE, Slack FJ: The role of microRNAs in cancer. *Yale J Biol Med* 2006;79:131-140.
- 23 Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultonwood J, Wainscoat JS, Hatton CS, Harris AL: Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol* 2008;141:672-675.
- 24 Liu R, Zhang C, Hu Z, Li G, Wang C, Yang C, Huang D, Chen X, Zhang H, Zhuang R, Deng T, Liu H, Yin J, Wang S, Zen K, Ba Y, Zhang CY: A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. *Eur J Cancer* 2011;47:784-791.
- 25 Qu Y, Zhang H, Duan J, Liu R, Deng T, Bai M, Huang D, Li H, Ning T, Zhang L, Wang X, Ge S, Zhou L, Zhong B, Ying G, Ba Y: MiR-17-5p regulates cell proliferation and migration by targeting transforming growth factor-beta receptor 2 in gastric cancer. *Oncotarget* 2016;7:33286-33296.
- 26 Zhang H: Cell-derived microvesicles mediate the delivery of miR-29a/c to suppress angiogenesis in gastric carcinoma. *Cancer Lett* 2016;375:331-339.

- 27 Li J, Liang H, Bai M, Ning T, Wang C, Fan Q, Wang Y, Fu Z, Wang N, Liu R, Zen K, Zhang CY, Chen X, Ba Y: miR-135b Promotes Cancer Progression by Targeting Transforming Growth Factor Beta Receptor II (TGFB2) in Colorectal Cancer. *PLoS One* 2015;10:e0130194.
- 28 Cheng Q, Feng F, Zhu L, Zheng Y, Luo X, Liu C, Yi B, Jiang X: Circulating miR-106a is a Novel Prognostic and Lymph Node Metastasis Indicator for Cholangiocarcinoma. *Sci Rep* 2015;5:16103.
- 29 Yao Y, Suo AL, Li ZF, Liu LY, Tian T, Ni L, Zhang WG, Nan KJ, Song TS, Huang C: MicroRNA profiling of human gastric cancer. *Mol Med Rep* 2009;2:963-970.
- 30 Yang K, Handorean AM, Iczkowski KA: MicroRNAs 373 and 520c are downregulated in prostate cancer, suppress CD44 translation and enhance invasion of prostate cancer cells in vitro. *Int J Clin Exp Pathol* 2009;2:361-369.
- 31 Liu P, Wilson MJ: miR-520c and miR-373 upregulate MMP9 expression by targeting mTOR and SIRT1, and activate the Ras/Raf/MEK/Erk signaling pathway and NF-kappaB factor in human fibrosarcoma cells. *J Cell Physiol* 2012;227:867-876.
- 32 Wu N, Song Y, Pang L, Chen Z: CRCT1 regulated by microRNA-520 g inhibits proliferation and induces apoptosis in esophageal squamous cell cancer. *Tumour Biol* 2016;37:8271-8279.
- 33 Yan L, Yu J, Tan F, Ye GT, Shen ZY, Liu H, Zhang Y, Wang JF, Zhu XJ, Li GX: SP1-mediated microRNA-520d-5p suppresses tumor growth and metastasis in colorectal cancer by targeting CTHRC1. *Am J Cancer Res* 2015;5:1447-1459.
- 34 Li R, Yuan W, Mei W, Yang K, Chen Z: MicroRNA 520d-3p inhibits gastric cancer cell proliferation, migration, and invasion by downregulating EphA2 expression. *Mol Cell Biochem* 2014;396:295-305.