

miR-494 inhibits cancer-initiating cell phenotypes and reverses resistance to lapatinib by downregulating FGFR2 in HER2-positive gastric cancer

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Abstract. In gastric cancer, >15% of cases are associated with the amplification of human epidermal growth factor receptor 2 (HER2), which leads to poor clinical outcomes. Lapatinib, a potent ATP-competitive inhibitor, is a small, orally active molecule, which inhibits the tyrosine kinases of HER2 and epidermal growth factor receptor type 1. The activation of receptor tyrosine kinases can contribute to lapatinib resistance in HER2-positive gastric cancer. The aim of the present study was to explore the effects of miR-494 and FGFR2 in regulation of cancer-initiating cell phenotypes and therapeutic efficiency of lapatinib in HER2-positive gastric cancer. Western blot analysis was used to identify that the expression of fibroblast growth factor receptor 2 (FGFR2), a receptor tyrosine kinase, was upregulated in gastric cancer tissues. Formation of cancer initiating cells (CICs) and resistance to lapatinib were determined using sphere growth assay and MTT assay, respectively. The overexpression of FGFR2 promoted the generation of cancer-initiating cells (CICs) and resistance to lapatinib in HER2-positive gastric cancer YCC1 cells. In addition, it was observed that overexpression of microRNA (miR)-494 downregulated the protein expression of FGFR2, inhibited the formation of CICs and reversed lapatinib resistance in YCC1-F cells (HER2-positive, FGFR2 overexpressing and lapatinib-resistant gastric cancer cells). Therefore, it was concluded that miR-494 inhibited the CIC phenotype and reversed resistance to lapatinib by inhibiting FGFR2 in HER2-positive gastric cancer.

Introduction

Gastric cancer is one of the most common types of malignant tumor and is the leading causes of cancer-associated mortality worldwide (1,2). The risk factors for this disease include diet, *Helicobacter pylori* infection and genetic alterations (3-5). It is reported that >95% of malignancies of the stomach are adenocarcinomas (6). The aggressiveness of human gastric cancer is associated with the activation of oncogenes, inactivation of tumor suppressor genes, and perturbation of growth factors and their receptors (7,8). However, the mechanisms controlling its level of aggression remain to be fully elucidated.

Human epidermal growth factor receptor 2 (HER2) is a proto-oncogene, which is encoded by ERBB2 on chromosome 17. Its amplification is detected in >15% of gastric cancer cases and is associated with poor clinical outcomes (9-14). Lapatinib (Tykerb; GlaxoSmithKline, Brentford, UK), a potent ATP-competitive inhibitor, is a small, orally active molecule, which inhibits the tyrosine kinases of HER2 and epidermal growth factor receptor type 1 (EGFR1) (15). Several studies have shown that the activation of receptor tyrosine kinases can mediate resistance to HER-targeted therapy (11). Fibroblast growth factor receptor 2 (FGFR2), a receptor tyrosine kinase, has been shown to be activated in several types of cancer through a variety of mechanisms, including gene amplification, translocations and point mutations (16). The expression of FGFR2 is increased in tumor tissues and positively correlated with clinicopathological factors; it promotes the invasion and migration of human gastric cancer cells (17,18).

Previous reports have shown that testican-1-mediated epithelial-mesenchymal transition signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer (19). In addition, the expression of phosphorylated (p-)MET, phosphorylated signal transducer and activator of transcription 3 (p-Stat3) and p-HER3 have been suggested as markers positively associated with resistance to lapatinib (19). Although these markers have been recognized, their regulatory mechanisms remain to be fully elucidated. The aim of the present study was to explore the effects of miR-494 and FGFR2 in regulation of cancer-initiating cell phenotypes and therapeutic efficiency of lapatinib in HER2-positive gastric cancer.

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Materials and methods

Clinical specimens. The Ethics Committees of Qilu Hospital of Shandong University (Jinan, China) approved the present study. Patient consent was obtained prior to tissue collection. In total, six gastric cancer samples and matched control tissue samples were frozen in liquid nitrogen immediately following surgical resection and were stored at -80°C until protein extraction. The surgical samples were obtained at Qilu Hospital of Shandong University between January 2015 and January 2017. The patients, 4 males and 2 females, ranged in age from 35-70 years, with a mean age of 61 years. According to American Joint Committee on Cancer clinical cancer stage (20), 2 patients were stage I/II, and 4 patients were stage III/IV. None of the patients received preoperative treatment, for example radiation or chemotherapy.

Cell culture. The YCC1 gastric cancer cell line (HER2-positive gastric cancer cells) and YCC1-F (HER2-positive, FGFR2 overexpressing and lapatinib-resistant gastric cancer cells) were obtained from Tiangen Biotech Co., Ltd. (Beijing, China). They were grown in RPMI-1640 medium (Sigma; Merck Millipore, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Shanghai, China) and 100 mg/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 .

Pre-miR-494/control miR and transfection. The pre-miR-494 and control miR were purchased from Ambion; Thermo Fisher Scientific, Inc. The cells were seeded at a density of 1.5×10^5 per well in 6-well plates or 60-mm dishes in 2 ml complete medium containing 10% FBS for 24 h. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For each well, the pre-miR-494 or negative control precursor miRNA (mock) was transfected into cells (Ambion; Thermo Fisher Scientific, Inc.). The mixture of Lipofectamine and miRNA (50 nM miR-494) was then administered to cells at 37°C in the presence of serum-free medium for up to 72 h.

Western blot analysis. Western blot analysis was performed as described previously (21). Total protein was prepared using extraction buffer comprising NaCl/ P_i containing 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). The concentration of each protein lysate was determined using a BCATM protein assay kit (Thermo Fisher Scientific, Inc.). Equal quantities (20 μg) of total protein were subjected to 12% SDS-PAGE. The samples were then transferred onto nitrocellulose membranes and blocked for 60 min at room temperature in 5% skim milk powder in NaCl/ P_i . The membranes were immunoblotted using antibodies against human FGFR2 (ab10648; 1:500), CD44 (ab157107; 1:500; Abcam), testican-1 (ab229935; 1:500; Abcam), MET (ab51067; 1:500; Abcam), HER2 (ab16901; 1:500; Abcam), Stat3 (ab119352; 1:500; Abcam), HER3 (ab32121; 1:500; Abcam), p-MET (ab5662; 1:500; Abcam), p-Stat3 (ab76315; 1:500; Abcam), p-HER3 (ab101407; 1:500; Abcam), c-myc (ab32072; 1:500; Abcam), insulin-like growth

factor 1 receptor (IGF1R; ab39398; 1:500; Abcam), or β -actin (ab5694; 1:500; all from Abcam, Cambridge, MA, USA) overnight at 4°C . IRDye[®] 800-conjugated anti-rabbit secondary antibody (ab191866; 1:10,000; Abcam) was used for incubation at room temperature for 30 min. The specific proteins were visualized using an OdysseyTM infrared imaging system (Gene Company, Ltd., Lincoln, NE, USA). The expression of β -actin was used as an internal control to ensure equal loading of the protein samples.

Sphere growth. The measurement of sphere growth was performed as described previously (22). Cells ($10^3/\text{ml}$) in serum-free RPMI-1640/1 mM Na-pyruvate were seeded on 0.5% agar precoated 6-well plates. After 10 days, half the medium was exchanged every third day. Single spheres were picked and counted. The sphere forming ability of the cells was recorded for the next 7-14 days. The size and number of spheres were assessed under a routine Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany).

MTT assay. To monitor resistance to Lapatinib, YCC1 and YCC1-F cells were treated with 1 μM Lapatinib for 24 h and then MTT assay was performed as described previously (19). Data were analyzed with Origin software version 7.5 (OriginLab, Northampton, MA, USA) to fit a sigmoidal curve. IC_{50} is the Lapatinib concentration that reduces proliferating cells by 50%.

Bioinformatics analysis. The analysis of potential miRNA target sites was performed using miRanda (<http://www.microrna.org/microrna/home.do>).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the cells by homogenizing cells in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (500 ng) was quantitated at 260 nm and reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's protocol, at 37°C for 15 min and 85°C for 30 sec.

For miRNA qPCR, reverse transcription was performed using the QuantMir RT kit (System Biosciences, Mountain View, CA, USA). cDNA was quantitated based on the absorption at 260 nm and served as the template for SYBR real-time PCR using Power SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). All reactions were run in triplicate on the iCycler iQ Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using miR-494-specific primers (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used: miR-494, forward 5'-TGGTGA TGGGATTTGAAACATACACGGGAAAC-3', and reverse 5'-AGATAGACGG-TGTCGCTGTTGAAGTCAG-3'; U6: Forward, 5'-GCTTCGGCAGCATACATATACTAA-3'; and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The amplification profile was as follows: Denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. The comparative cycle quantification (Cq) method was used to

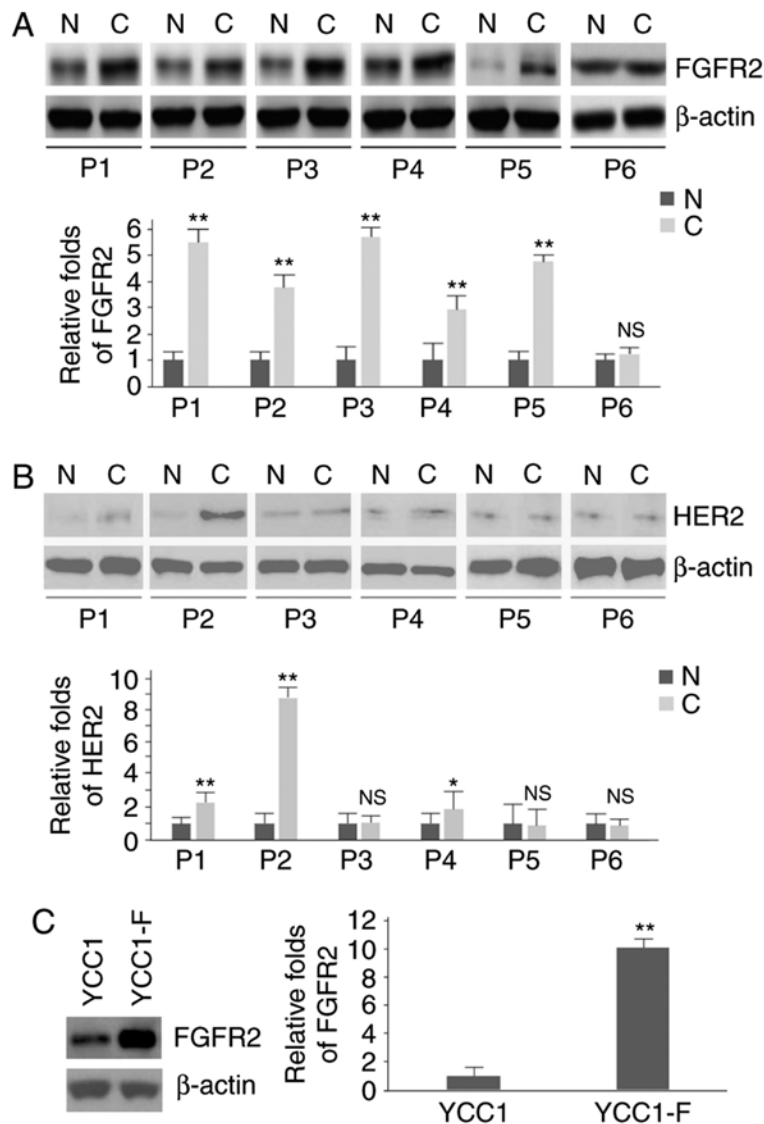


Figure 1. Protein expression of FGFR2 is increased in gastric cancer tissues and YCC1-F cells. (A) Western blotting of FGFR2 protein in gastric cancer tissues and adjacent normal tissues (n=6). Bar chart shows the quantified data for the blots. (B) Western blot of HER2 protein in gastric cancer tissues and adjacent normal tissues (n=6). Bar chart shows the quantified data for the blots. (C) Western blot of FGFR2 protein in YCC1 and YCC1-F cells (n=3). Bar chart shows the quantified data for the blots. *P<0.05 and **P<0.01 vs. control. NS, no significant difference; C, gastric cancer tissue; N, normal tissue; P1-6, patients 1-6. FGFR2, fibroblast growth factor receptor 2; HER2, human epidermal growth factor receptor 2.

quantify the miRNA expression levels. The relative quantity of miR-494 to small nuclear U6 RNA was calculated using the $2^{-\Delta C_q}$ equation, where $\Delta C_q = (C_{q_{miR-494}} - C_{q_{U6 RNA}})$. The fold change of gene expression was calculated using the $2^{-\Delta \Delta C_q}$ method (23). U6 small nuclear RNA was used as the internal standard.

For mRNA qPCR, analysis was performed as described above. cDNA was quantitated based on the absorption at 260 nm and served as the template for qPCR. The thermal cycle profile was as follows: Denaturation for 30 sec at 95°C, annealing for 45 sec at 52-58°C depending on the primers used, and extension for 45 sec at 72°C. Each PCR reaction was performed for 28-32 cycles. PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV transillumination. RT-qPCR was performed using a StepOne™ real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Fast SYBR®-Green Master

Mix was also obtained from Applied Biosystems. Data are shown as a relative expression level after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: FGFR2, forward 5'-GGTCGT TTCATCTGCCTGGT-3' and reverse 5'-CCTTCCCGTTTT TCAGCCAC-3'; CD44, forward 5'-CAGCAACCCTACTGA TGATGACG-3' and reverse 5'-GCCAAGAGGGATGCCAAG ATGA-3'; GAPDH, forward 5'-CGGAGTCAACGGATTTGG TCGTAT-3' and reverse 5'-AGCCTTCTCCATGGTGGTGAA GAC-3'.

Statistical analysis. The results were analyzed using SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA) (24). Data are presented as the mean \pm standard error of the mean of separate experiments (n=3). Statistical significance was determined using Student's t-test (two-tailed). P<0.05 was considered to indicate a statistically significant difference.

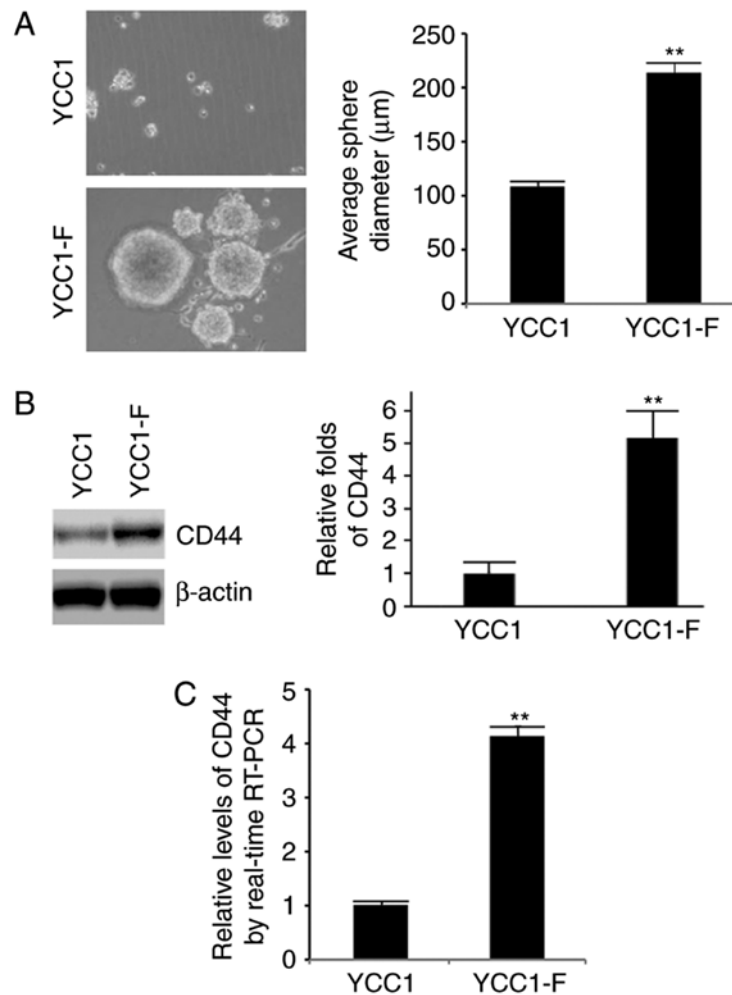


Figure 2. Overexpression of FGFR2 is associated with increased formation of CICs. (A) Sphere growth for YCC1 and YCC1-F cells (original magnification, $\times 100$, $n=3$). (B) Western blotting of CD44 protein in YCC1 and YCC1-F cells ($n=3$). Bar chart shows the quantified data for the blots. (C) Reverse transcription-quantitative polymerase chain reaction analysis of CD44 mRNA in YCC1 and YCC1-F cells ($n=3$). ** $P<0.01$ vs. control. FGFR2, fibroblast growth factor receptor 2; CICs, cancer initiating cells.

Results

FGFR2 protein is increased in gastric cancer tissues and YCC1-F cells. To identify the protein expression FGFR2 and HER2 in gastric cancer tissues and their adjacent normal tissues, western blot analysis was performed in six pairs of gastric cancer tissues and their adjacent normal tissues. It was observed that the protein expression of FGFR2 was significantly increased in five tumor tissues (patients 1-5; Fig. 1A).

Statistically significant differences in the protein expression of HER2 were observed between the gastric cancer tissues and their adjacent normal tissues in patient 1, patient 2 and patient 4 (Fig. 1B). In addition, western blot analysis was performed to determine the protein expression of FGFR2 in YCC1 cells (HER2-positive gastric cancer cells) and YCC1-F cells (HER2-positive and FGFR2-overexpressing gastric cancer cells). The results showed that the protein level of FGFR2 was upregulated in the YCC1-F cells (Fig. 1C).

FGFR2 induces the formation of cancer-initiating cell (CIC) phenotypes. To determine whether FGFR2 affects CICs, a sphere-forming assay was performed to assess the formation of stem cell-like populations. It was found that the formation of

spheres was increased in the YCC1-F cells (Fig. 2A). CD44 is a robust marker and is of functional importance for CICs (25). In order to detect whether the expression of CD44 can be affected by FGFR2, western blot and RT-qPCR analyses were performed. It was observed that the protein and mRNA expression levels of CD44 were upregulated in the YCC1-F cells (Fig. 2B and C).

Overexpression of FGFR2 promotes resistance to lapatinib.

In order to determine whether FGFR2 can affect the efficacy of lapatinib, an MTT assay was performed in the treated YCC1 and YCC1-F cells (Fig. 3A). The results showed that the overexpression of FGFR2 promoted resistance to lapatinib (Fig. 3A). The expression of testican-1, HER3, p-HER3, MET, p-MET and p-Stat3 has been suggested as markers positively associated with lapatinib resistance in HER2-positive gastric cancer (19). Therefore, the present study performed western blot analysis to examine the protein expression levels of testican-1, MET, HER2, Stat3, HER3, p-MET, p-Stat3 and p-HER3 in the YCC1 and YCC1-F cells. It was observed that the protein expression levels of testican-1, p-MET, p-Stat3 and p-HER3 were increased by FGFR2 (Fig. 3B and C).

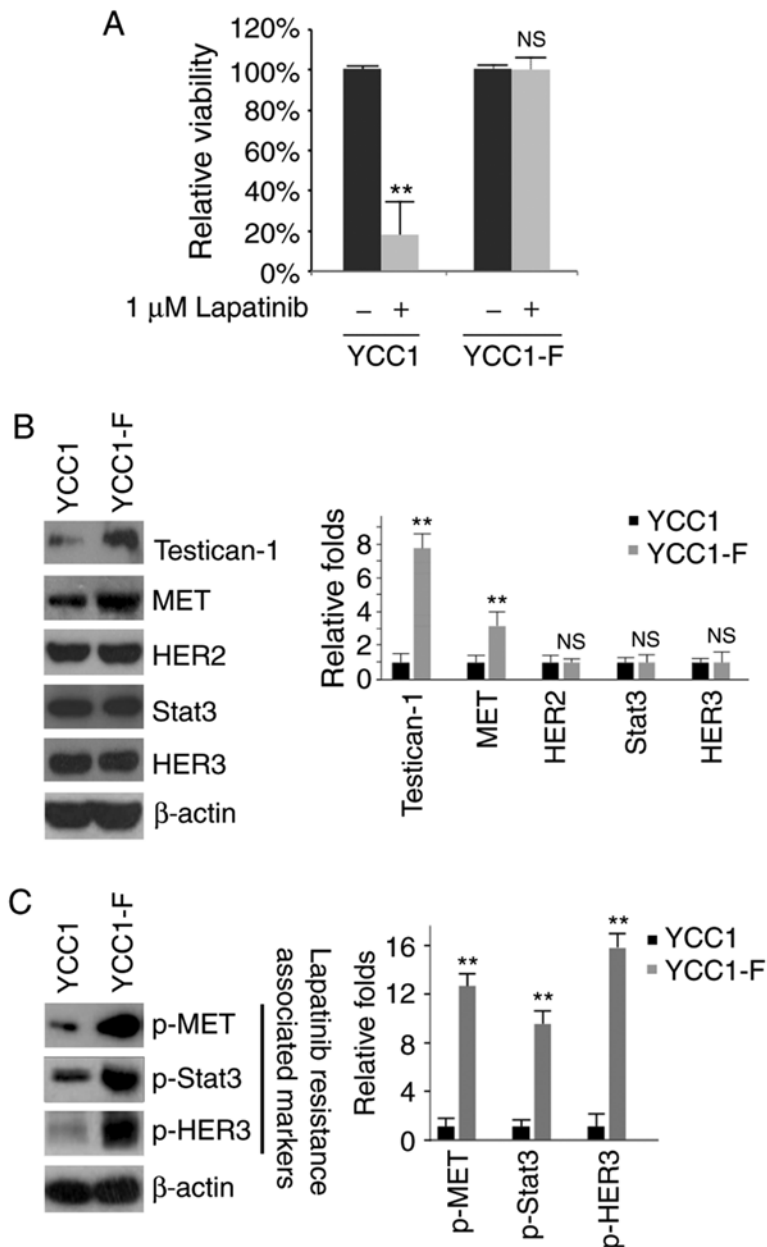


Figure 3. Overexpression of FGFR2 promotes resistance to lapatinib in YCC1 cells. (A) MTT assay of cell viability in YCC1 and YCC1-F cells with or without treatment with lapatinib (n=3). (B) Western blot analysis of testican-1, MET, HER2, Stat3 and HER3 proteins in YCC1 and YCC1-F cells (n=3). Bar chart shows the quantified data for the blots. (C) Western blot analysis of p-MET, p-Stat3 and p-HER3 proteins in YCC1 and YCC1-F cells (n=3). Bar chart shows the quantified data for the blots. **P<0.01 vs. control. NS, no significant difference; FGFR2, fibroblast growth factor receptor 2; HER, human epidermal growth factor receptor; Stat3, signal transducer and activator of transcription 3; p-, phosphorylated.

miR-494 inhibits the expression of FGFR2 in YCC1-F cells. The present study also aimed to investigate the molecular mechanism regulating the expression of FGFR2 in YCC1-F cells. miRNAs are an emerging class of small, non-coding, single-stranded RNAs, which serve as important regulators of gene expression by binding to the 3' untranslated region (UTR) of target mRNAs, leading to their translational repression and/or degradation (26-28). To examine whether FGFR2 can be regulated by miRNAs, the present study used miRanda, a commonly used prediction algorithm (<http://www.microrna.org/microrna/home.do>) to analyze the 3'UTR of FGFR2. In total, 39 miRNAs were found using the algorithm: miR-494; miR-374b; miR-374a; miR-590-3p; miR-217; miR-381; miR-300; miR-103; miR-107;

miR-15b; miR-424; miR-15a; miR-497; miR-16; miR-195; miR-223; miR-410; miR-543; miR-153; miR-431; miR-485-5p; miR-194; miR-544; miR-382; miR-33a; miR-33b; miR-22; miR-125a-5p; miR-125b; miR-542-3p; miR-330-5p; miR-326; miR-429; miR-200b; miR-200c; miR-145; miR-340; miR-218 and miR-296-3p. However, the present study focused on miR-494, as miR-494 is a candidate tumor suppressor gene (29). It was hypothesized that miR-494 downregulates the protein expression of FGFR2 by targeting its 3'UTR in YCC1-F cells. The target sites on the 3'UTR of FGFR2 are shown in Fig. 4A.

To examine the expression of miR-494 in the YCC1 and YCC1-F cells, RT-qPCR analysis was performed. It was found that miR-494 was decreased in the YCC1-F cells (Fig. 4B). To

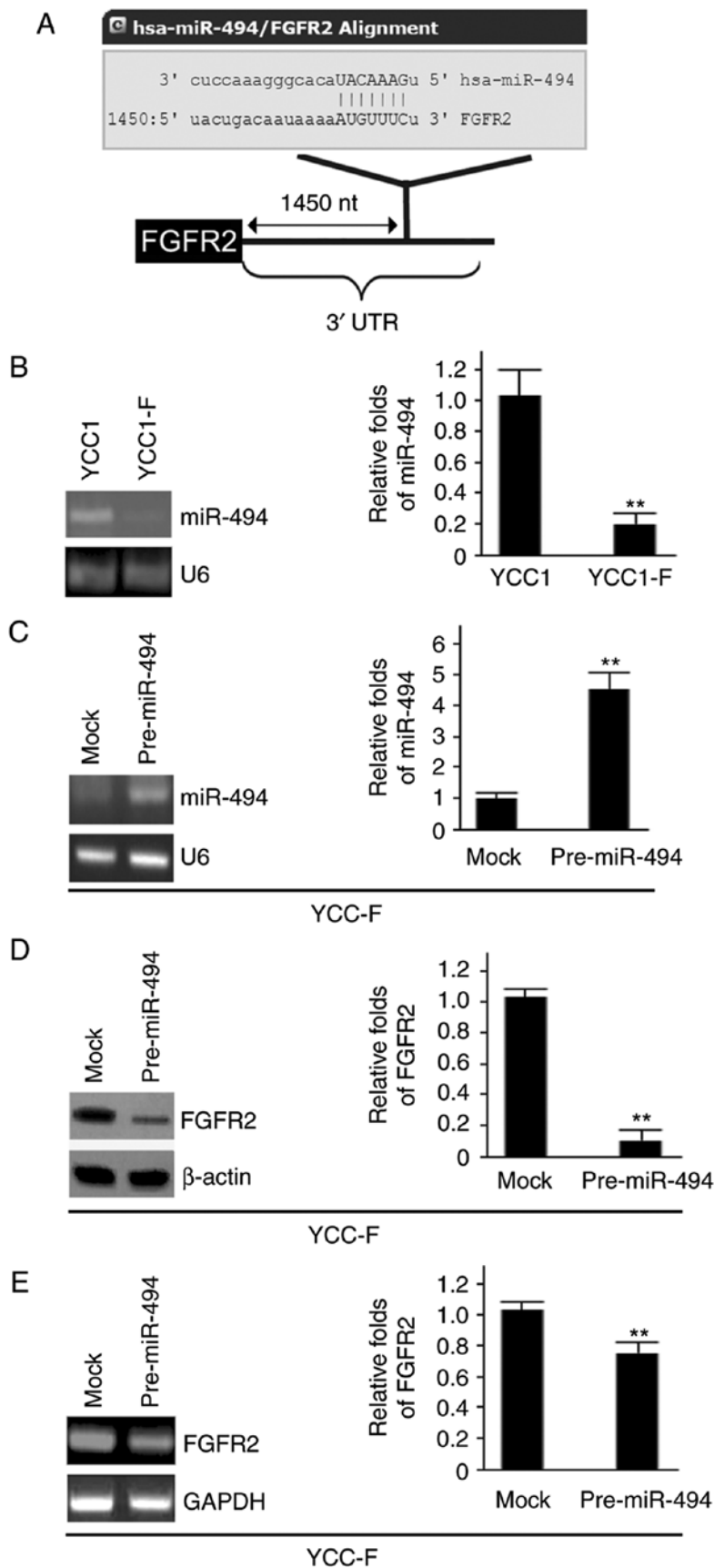


Figure 4. miR-494 inhibits the expression of FGFR2 in YCC1-F cells. (A) Schematic of the predicted miR-494 binding sites in the 3'UTR of FGFR2 mRNA by miRanda. (B) RT-qPCR analysis of miR-494 in YCC1 and YCC1-F cells. U6 was a loading control (n=3). Bar chart shows the quantified data for the blots. (C) RT-qPCR analysis of miR-494 in YCC1-F cells transfected with pre-miR-494 and control (mock) miR (n=3). Bar chart shows the quantified data for the blots. (D) Western blot analysis of FGFR2 protein in YCC1-F cells transfected with pre-miR-494 or control (mock) miR (n=3). Bar chart shows the quantified data for the blots. (E) Results of RT-qPCR analysis of FGFR2 mRNA in YCC1-F cells transfected with pre-miR-494 or control (mock) miR (n=3). Bar chart shows the quantified data for the blots. **P<0.01 vs. control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; FGFR2, fibroblast growth factor receptor 2; 3'UTR, 3' untranslated region.

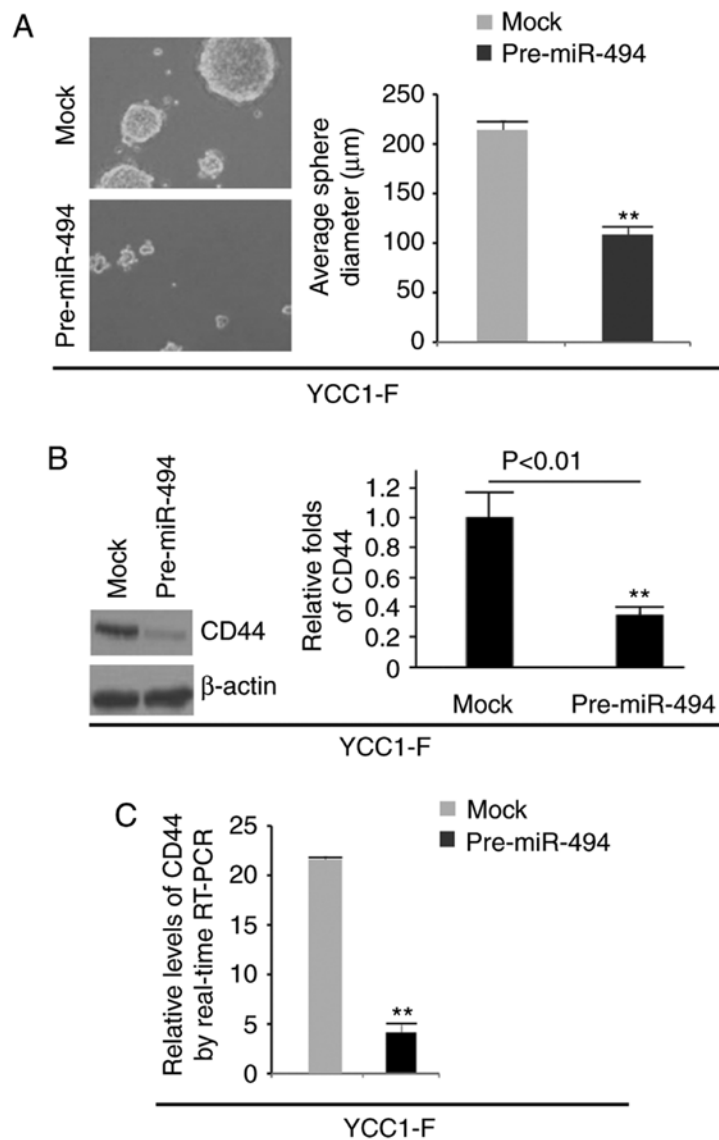


Figure 5. miR-494 inhibits the formation of CICs in YCC1-F cells. (A) Sphere growth for YCC1-F cells transfected with pre-miR-494 or control (mock) miR (original magnification, $\times 100$, $n=3$). (B) Western blot analysis of CD44 protein in YCC1-F cells transfected with pre-miR-494 or control (mock) miR ($n=3$). Bar chart shows the quantified data for the blots. (C) RT-qPCR analysis of CD44 mRNA in YCC1-F cells transfected with pre-miR-494 or control (mock) miR ($n=3$). ** $P<0.01$ vs. control. miR, microRNA; CICs, cancer initiating cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

examine the effect of miR-494, YCC1-F cells were transfected with pre-miR-494 and control miR, and the expression of miR-494 was examined using RT-qPCR analysis. It was found that miR-494 was increased by pre-miR-494 in the YCC1-F cells (Fig. 4C). To determine whether miR-494 regulates the protein expression of FGFR2, western blot analysis was performed to examine the protein expression of FGFR2 in YCC1-F cells transfected with pre-miR-494 and control miR. It was observed that FGFR2 protein was significantly inhibited by miR-494 (Fig. 4D). RT-qPCR analysis was then performed to examine the mRNA expression of FGFR2 in the YCC1-F cells transfected with pre-miR-494 and control miR. It was found that the mRNA level of FGFR2 was inhibited by miR-494 (Fig. 4E).

miR-494 inhibits the formation of CIC phenotypes in YCC1-F cells. To determine whether miR-494 can affect the formation of CICs, a sphere-forming assay was performed to assess the

formation of stem cell-like populations. It was found that the formation of spheres was decreased in the YCC1-F cells transfected with pre-miR-494 (Fig. 5A). To determine whether the expression of CD44 was affected by miR-494, western blot and RT-qPCR analyses were performed to examine its expression. The results showed that the protein and mRNA levels of CD44 were downregulated in the YCC1-F cells transfected with pre-miR-494 (Fig. 5B and C).

miR-494 reverses lapatinib-resistance in YCC1-F cells. To determine whether miR-494 affects lapatinib efficacy, an MTT assay was performed of the YCC1-F cells following treatment (Fig. 6A). The results showed that the overexpression of miR-494 reversed resistance to lapatinib (Fig. 6A). Western blot analysis was also performed to detect the protein expression levels of testican-1, MET, HER2, Stat3, HER3, p-MET, p-Stat3 and p-HER3 in the YCC1-F cells. The results showed that the protein expression levels of testican-1,

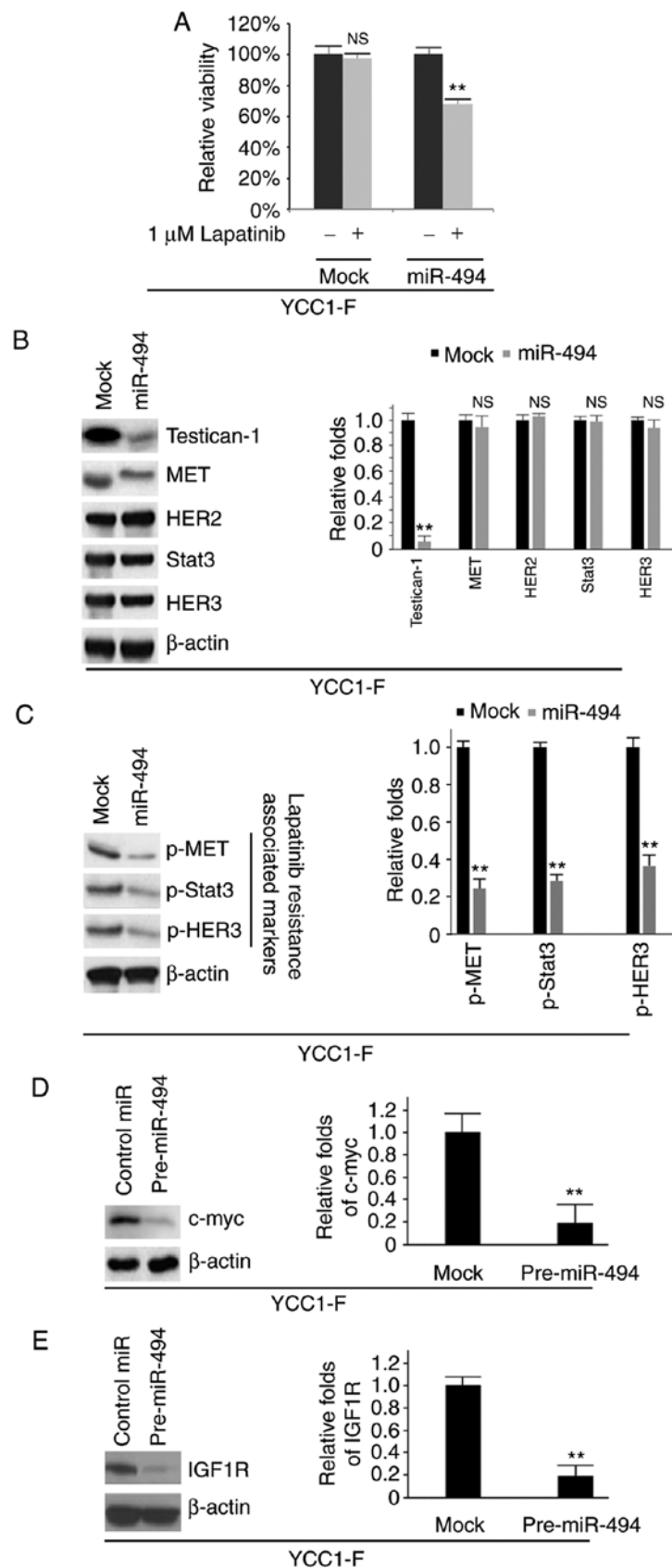


Figure 6. miR-494 reverses resistance to lapatinib in YCC1-F cells. (A) MTT assay of YCC1-F cells. YCC1-F cells transfected with pre-miR-494 and control miR (mock) were untreated or treated with lapatinib (n=3). (B) Western blot analysis of testican-1, MET, HER2, Stat3 and HER3 proteins in YCC1-F cells transfected with pre-miR-494 or control (mock) miR (n=3). Bar chart shows the quantified data for the blots. (C) Western blot analysis of p-MET, p-Stat3 and p-HER3 proteins in YCC1-F cells transfected with pre-miR-494 or control (mock) miR (n=3). Bar chart shows the quantified data for the blots. (D) Western blot analysis of c-myc protein in YCC1-F cells transfected with pre-miR-494 and control miR (n=3). Bar chart shows the quantified data for the blots. (E) Western blot analysis of IGF1R protein in YCC1-F cells transfected with pre-miR-494 and control miR (n=3). Bar chart shows the quantified data for the blots. **P<0.01 vs. control. NS, no significant difference; miR, microRNA; HER, human epidermal growth factor receptor; Stat3, signal transducer and activator of transcription 3; p-, phosphorylated; IGF1R, insulin-like growth factor 1 receptor.

p-MET, p-Stat3 and p-HER3 in the YCC1-F cells were inhibited by miR-494 (Fig. 6B and C). To investigate whether the expression of c-myc and IGF1R were regulated by miR-494 in YCC1-F cells, western blot analysis was performed to determine the protein expression of c-myc and IGF1R in cells transfected with pre-miR-494 or control miR. It was observed that the overexpression of miR-494 significantly inhibited the protein expression of c-myc and IGF1R in the YCC1-F cells (Fig. 6D and E).

Discussion

The frequency of overexpression was reported as 11.8% for HER2 and 31.1% for FGFR2 in a large cohort of patients with gastric cancer (30). HER2-positive gastric cancer exhibits more differentiated tumor types (papillary and tubular adenocarcinoma) and is more frequently associated with venous invasion and regional lymph node metastasis, compared with HER2-negative cancer (30). Similar to HER2-positive gastric cancer, FGFR2-positive gastric cancer is more frequently associated with vascular invasion and a more advanced tumor stage, compared with FGFR2-negative gastric cancer (30). In the present study, it was observed that the protein expression of FGFR2 was significantly increased in five of six tumor tissues examined, and statistically significant differences in the protein expression of HER2 were observed between gastric cancer tissues and their adjacent normal tissues in three of the six patients.

Small molecule inhibitors of HER2, including lapatinib, are clinically active in women with advanced HER2-positive gastric cancer (11). However, the effectiveness of this class of agent is limited by either primary resistance or acquired resistance. The molecular mechanisms underlying the resistance of HER2-positive gastric cancer cells to lapatinib remain to be fully elucidated. The activation of receptor tyrosine kinases can contribute to lapatinib resistance in HER2 positive gastric cancer (11). FGFR2 is a receptor tyrosine kinase (31), and its overexpression promotes the expression of CD44 and accelerates tumor growth in mice, and can maintain stemness in gastric cancer (32). In the present study, it was observed that FGFR2 was increased in gastric cancer and this overexpression promoted the formation of CICs, consistent with the findings of a previous report (32). This increased formation of CICs can lead to drug resistance (33-35). In accordance with previous reports (33-35), the present study found that the overexpression of FGFR2 contributed to lapatinib resistance in gastric cancer.

Testican-1 can confer acquired resistance to lapatinib (19). The results of the present study showed that testican-1 was upregulated by FGFR2 protein in the HER2-positive gastric cancer cells. The expression levels of p-MET, p-Stat3, p-HER3 are positively associated with resistance to lapatinib (19). The present study found that FGFR2 promoted the protein expression of p-MET, p-Stat3 and p-HER3 in the HER2-positive gastric cancer cells. These results suggested that FGFR2 contributes to lapatinib resistance by regulating the protein expression levels of testican-1, p-MET, p-Stat3 and p-HER3 in HER2-positive gastric cancer cells. Lapatinib, a potent ATP-competitive inhibitor, is a small, orally active molecule, which inhibits HER2 and EGFR (15). One of limitations of

the present study was the lack of HER2 and EGFR antibody expression.

The expression of miR-494 is decreased in gastric cancer and acts as an anti-oncogene (36). The present study found that overexpressing miR-494 downregulated the expression of FGFR2. In contrast to the role of FGFR2, it was shown that miR-494 inhibited the formation of CICs. In addition, it was observed that overexpressing miR-494 reversed resistance to lapatinib. It was hypothesized that miR-494 regulates the expression of FGFR2 by targeting its 3'UTR in gastric cancer, and that the downregulation of miR-494 may contribute to the upregulation of FGFR2, promoting resistance to lapatinib in HER2-positive gastric cancer. miR-494 acts as a tumor suppressor gene in gastric cancer by targeting c-myc and IGF1R (36,37). Consistent with previous reports (36,37), the present study confirmed that the c-myc and IGF1R proteins were inhibited by miR-494. These results suggested that the decrease of miR-494 may be causal in the upregulated protein expression of FGFR2.

Taken together, the results of the present study provide novel insights for understanding the stemness phenotype and resistance to lapatinib developed by HER2-positive gastric cancer cells, which may contribute to the future development of novel anti-gastric cancer strategies.

Competing interests

The authors declare that they have no competing interests.

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