Downregulation of microRNA-155 accelerates cell growth and invasion by targeting c-myc in human gastric carcinoma cells

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Abstract. MicroRNAs (miRNAs) are a recently discovered class of small non-coding RNAs that regulate gene expression. miRNAs can contribute to cancer development and progression and are differentially expressed in normal tissue and cancer. In the present study, our aim was to investigate the expression of miR-155 in gastric cancer and to explore the mechanisms by which it influences gastric cancer cells. The level of miR-155 in 52 gastric carcinoma and corresponding non-tumor tissues was quantified by real-time reverse transcriptase-polymerase chain reaction. We used the data from EdU, CASY and cell adhesion assays to show how the expression of miR-155 affects viability and proliferation in SGC-7901 cancer cells. We also performed functional assays using the 3'-untranslated region (3'-UTR) of the c-myc gene as a miR-155 target in a luciferase reporter assay system. Our results indicated that miR-155 is downregulated in both human gastric carcinoma tissues and SGC-7901 cells. The high expression level of miR-155 may significantly downregulate cancer cell viability, proliferation and attachment. The level of miR-155 could influence endogenous c-myc expression in SGC-7901 cells, and may decrease its expression by binding to 3'-UTR of c-myc. In conclusion, our results suggest that miR-155 is extensively involved in the cancer pathogenesis of gastric carcinoma and support its function as recessive cancer genes. c-myc is an important miR-155 target gene.

Introduction

MicroRNAs (miRNAs) are a recently discovered class of small non-coding RNAs that regulate gene expression (1). Mature miRNAs are the results of sequential processing of primary transcripts (pri-miRNAs) mediated by two RNase III enzymes, Drosha and Dicer (2). Mature 18-24 mer long miRNAs negatively regulate protein expression of specific mRNAs by either translational inhibition or mRNA degradation (3). miRNAs are differentially expressed in various human cancers. A number of previous reports have provided a considerable amount of support for a critical role of many miRNAs in cancer (4-6). Strong correlations between miRNA expression profiles and specific cancer lineages have been observed (for reviews,7,8), and that may be due to their fundamental importance in regulatory processes involved in establishing and maintaining the tumor phenotype. Further support for their role in tumorigenesis may come from genetic evidence. Notably, Calin *et al* showed that miRNAs are often encoded in fragile sites in the genome, where their expression can be altered by events such as genomic amplification, loss of heterozygosity, viral integration or genomic rearrangement (9). Another study also found that miRNAs display a high frequency of genomic alterations in human cancers (10). Array profiling studies have shown strong correlations between the expression of specific miRNAs and the tumor phenotype (11-14). Further evidence for an oncogenic role of certain miRNAs came from transgenic mouse studies showing that overexpression of the miR-19 cluster or miR-155 results in increased frequency of tumor formation (15,16).

miR-155, which is overexpressed in breast, lung and colon cancer (17,18), was also previously shown to be involved in numerous cellular processes including proliferation, differentiation, apoptosis and metabolism. However, since it is strongly upregulated in normal pancreatic tissue, when an all tumors vs. all normal comparison is performed, miR-155 appears as downregulated in solid tumors, albeit with a borderline significance (19). In the present study, we determined the expression of mir-155 in gastric cancer and its functional role in gastric cancer cells (SGC-7901). We used fluorescence-based quantitative real-time PCR as well as precursor molecules to investigate the expression level of miR-155 in human gastric carcinoma and normal tissue. Due to the considerable evidence that miRNAs are involved in cancer (20-22), it is key to elucidate their roles in solid tumors and to further elucidate common miRNA-driven pathways; we also looked for downstream targets of miR-155 in SGC-7901 gastric cancer cells in order to ascertain the relationship between mir-155 and the pathogenesis of gastric cancer.

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Materials and methods

Patients and specimens. All human tissue samples were obtained from surgical specimens of 52 patients with gastric carcinoma from 2006 to 2007 at the Second Affiliated Hospital, Harbin Medical University, China. All tissues, including gastric carcinoma and corresponding adjacent normal tissue, were divided into two parts and preserved in liquid nitrogen for 30 min after removing from the body. All of the samples were obtained with patient's informed consent and were histologically confirmed.

Cell culture. SGC-7901 (human gastric cancer cell line) and HEK293 (human embryonic kidney cell line) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen) plus 0.5% penicillin-streptomycin at 37˚C in a 5% CO_2 incubator.

Quantitative reverse transcription-PCR. Real-time quantitative PCR was performed using standard protocols on an Applied Biosystem 7500 HT Sequence Detection System. All reagents and primers (miRNAs155, 5s) were purchased from Ambion. Briefly, 5 μl of a 1/100 dilution of cDNA in water was added into 12.5 μl of the 2X SYBR-Green PCR master mix (Ambion), with 800 nmol/l of each primer in a total volume of 25 μl. The reactions were amplified for 15 sec at 95˚C, and 1 min at 60˚C for 40 cycles. All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined for each gene, and the relative amount of each miRNA to 5sRNA was calculated using the equation 2-ΔCT, where ΔCT = (CTmiRNA - CT5s) (23).

Oncogene target predictions. The recent Sanger predictions (April 2008) were used to identify the putative miRNA targets. They included essentially the 3'-untranslated region (3'-UTR) targets reported by Lewis *et al* (24) with a few changes arising from updated gene boundary definitions from the April 2005 UCSC Genome Browser mapping of RefSeq mRNAs to the hg17 human genome assembly. Among the putative targets, we specified known cancer genes (tumor suppressors and oncogenes) as identified in the Cancer Gene Census at http://www.pubmedcentral.nih.gov/redirect3.cs, or reported by OMIM at http://www.pubmedcentral.nih.gov/redirect3 (19). We obtained the network of miR-155 regulation by RG Bioconductor.

Target in vitro assays. For luciferase reporter experiments, the 3'-UTR segments (Invitrogen) of myc predicted to interact with miR-155 were annealed synthetic from myc cDNA (ENST00000377970) and ligated into the pMIR-REPORT™ vector (Ambion), using the *Spe*I and *Hind*III sites immediately downstream from the stop codon of luciferase. The HEK293 cell line was grown in 10% FBS in RPMI-1640 medium, supplemented with 1X non-essential amino acid and 1 mmol sodium pyruvate at 37˚C in a humidified atmosphere of 5% CO_2. The cells were cotransfected in 12-well plates by using siPORT NeoFx (Ambion), according to the manufacturer's protocol, with 0.4 μg of the firefly luciferase report vector and galactosidase activity was used for normalizing the transfection efficiency and protein input. For each well, 10 or 50 nM miRNA oligonucleotides or scrambled oligonucleotides (both from Ambion) were used. Firefly and *Renilla* luciferase activities were measured consecutively by using Dual-Luciferase assays (Promega) 24 h after transfection.

Transfection of miR-155. Transfection of SGC-7901 cells with miR155 mimic or the negative control oligonucleotide (both from Ambion) by siPORT NeoFX (Ambion) was performed according to the manufacturer's protocol. SGC-7901 cells were seeded in 6-well plates at a concentration of $1x10^5$/well. The effects caused by the introduction miR-155 mimic into the cells were assayed at 48 h after the transfection by western blot analyses for the protein of myc.

Cell adhesion assays. Fibronectin (Sigma, Beijing, China) was dissolved in phosphate-buffered saline (PBS) to the 2 μg/50 μl solution, then coated to each well of 96-well plates with the solution for 100 μl, dried in horizontal laminar flow clean workbench overnight. The plates were washed with PBS and blocked with RPMI-1640 medium (50 μl/well) for 1 h before use. SGC-7901 cells were cultured in the presence of miR-155 mimics or miR-control (0, 30, 50, 80 or 100 nM) for 24 h and then resuspended in serum free medium. The cell concentration was adjusted to $6x10^8$ cells/l. The fibronectin coated wells were seeded with 100 μl of this cell suspension and incubated for 2 h at 37˚C. The medium was discarded and the wells were washed with 100 μl of pre-warmed PBS to wash out the unattached cells. DMSO (100 μl) was added to each well after 4 h incubation by 100 μl MTT (1 mg/ml). The OD values were measured by a microplate reader as described above.

EdU proliferation assay and cell viability. Proliferation was determined *in vitro* using the EdU DNA proliferation *in vitro* detection kit (RiboBio, China), according to the manufacturer's instructions. Proliferating SGC-7901 cells after transfection by miR-155 mimics were incubated with 50 μm EdU for 2 h, then fixed and permeated by PBS (respectively containing 4% polyoxymethylene, 0.5% Triton X-100), and cell nuclei were stained with Apollo and Hoechst staining solution at an advisable concentration for 30 min, observed by confocal laser scanning microscope (CLSM) and the result was analyzed by IPP6.0 software (Image-Pro Plus).

SGC-7901 cells were harvested at 24 h after transfection with miR-155 mimics (30, 50, 80 and 100 nm). The cell viability was obtained by CASY measurement (CASY-1; Schärfe System, Reutlingen, Germany), according to the manufacturer's instructions.

Western blotting. Antibody samples, myc and β-actin, (purchased from Santa Cruz) were lysed in buffer (1% NP-40, 0.1 M Tris, pH 8.0, 0.15 MNaCl, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). The protein pellets were resuspended in the buffer, followed by pH adjustment. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membrane with a semidry electrophoresis transfer apparatus (Bio-Rad) and probed with antibodies according to the manufacturer's instructions.

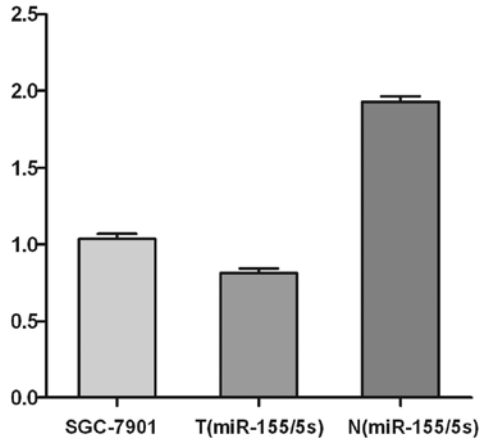

Figure 1. Level of miR-155 in gastric carcinoma tissues (n=32) and SGC-7901 was lower than in non-tumor tissues (n=32). Values shown (ΔCt) are relative to those of 5s small RNA.

Statistical analysis. All data are reported as means ± SEM. When comparisons were made between two different groups, statistical analysis was performed by the Student's t-test and ANOVA. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed with the SPSS 19.0 statistics software (SPSS Inc., Chicago, IL, USA).

Results

Expression level of miR-155 is downregulated in SGC-7901 cells and gastric cancer samples. In order to confirm whether the level of miR-155 was reduced in human gastric cancer, we examined the expression of mature miR-155 and 5s in the samples from 52 patients with gastric cancer as well as in SGC-7901 cells. As shown in Fig. 1, the expression level of miR-155 miRNA in tumors was significantly lower (downregulation rate >2-fold) in SGC-7901 cells and 32 patients tested. However, in the other 20 patients, no significant difference in miR-155 expression was observed between tumors and normal tissues, and the up- or downregulation rates were within 2-fold.

Overexpression of miR-155 reduces SGC-7901 cell proliferation and viability. Using CASY and EdU assay, we evaluated the function of high level miR-155 in gastric carcinoma cells by measuring cell viability and proliferation in SGC-7901 cells which were transfected with the miR-155 mimic of different concentrations. CASY measurement showed a significant decrease in the viability of SGC-7901 cells transfected by miR-155 mimics when compared with the negative control group. Cells treated with 50 nM or higher concentrations of miR-155 showed significantly lower rates of viability when compared with control miRNA-treated cells (Fig. 2A).

Cell adhesion assays (Fig. 2B) showed similar results; high level of mir-155 reduced the adhesion ability, and the inhibition rate correspondingly increased according to the increased concentration of mir-155 mimics. The mir-155 mimics 100 nm group showed the most obvious change when compared with the control groups.

The result of the EdU assay (Fig. 3) also showed the level of miR-155 modulated SGC-7901 cell proliferation. EdU experiments were performed to assess proliferation after SGC-7901 cells were transfected with mir-155 inhibitor or mimics (100 nm). Under these conditions, the level of proliferation in the miR-155 mimics-transfected cells was reduced, compared with cells transfected with the control group. On the contrary, compared with cells in the control group, proliferation of SGC-7901 cells which were transfected by miR-155 inhibitor was increased. The results support the theory that high level of miR-155 is able to prevent SGC-7901 proliferation.

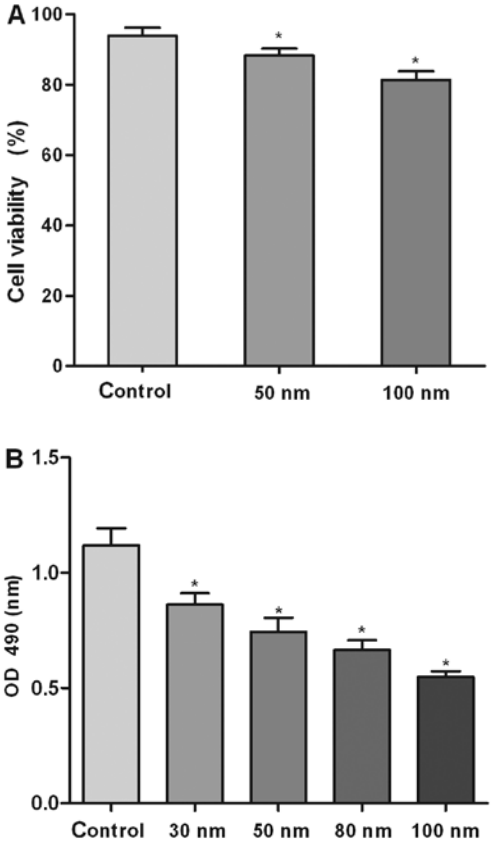

Figure 2. (A) Viability of cells transfected with miR-control or miR-155 mimics (50 and 100 nM). (B) Adhesion assay shows the ability of cells to attach to fibronectin. *P<0.05, indicates a significant difference was observed as compared to the control group in concentration 0 nM.

Target assay of miR-155. The functional significance of miR-155 downregulation in cancer shown here needs to be understood. First, *in silico* prediction of targets was performed by using Sanger database (version 5) of conserved 3'-UTR miRNA targets (13). Sanger contained 991 predictions for miR-155. Some well known cancer genes were predicted as targets for miR-155 (Fig. 4A). We then experimentally confirmed the *in silico* predictions for one cancer gene, myc (proto-oncogene protein). To experimentally validate the computational data, the myc 3'-UTR (2,356 bp) was subcloned downstream of the f-luc open reading frame (*Hind*III and *Spe*I sites). This reporter construct (pMIR/2356/5'3') was cotransfected in the HEK293 cell line with pMIR-REPORT™ (to normalize for transfection differences) and a control non-targeting RNA oligonucleotide (miR-control) or miR155 precursor RNA oligonucleotide (Ambion). The relative luciferase activity was markedly diminished (61.3±4.2%) in cells

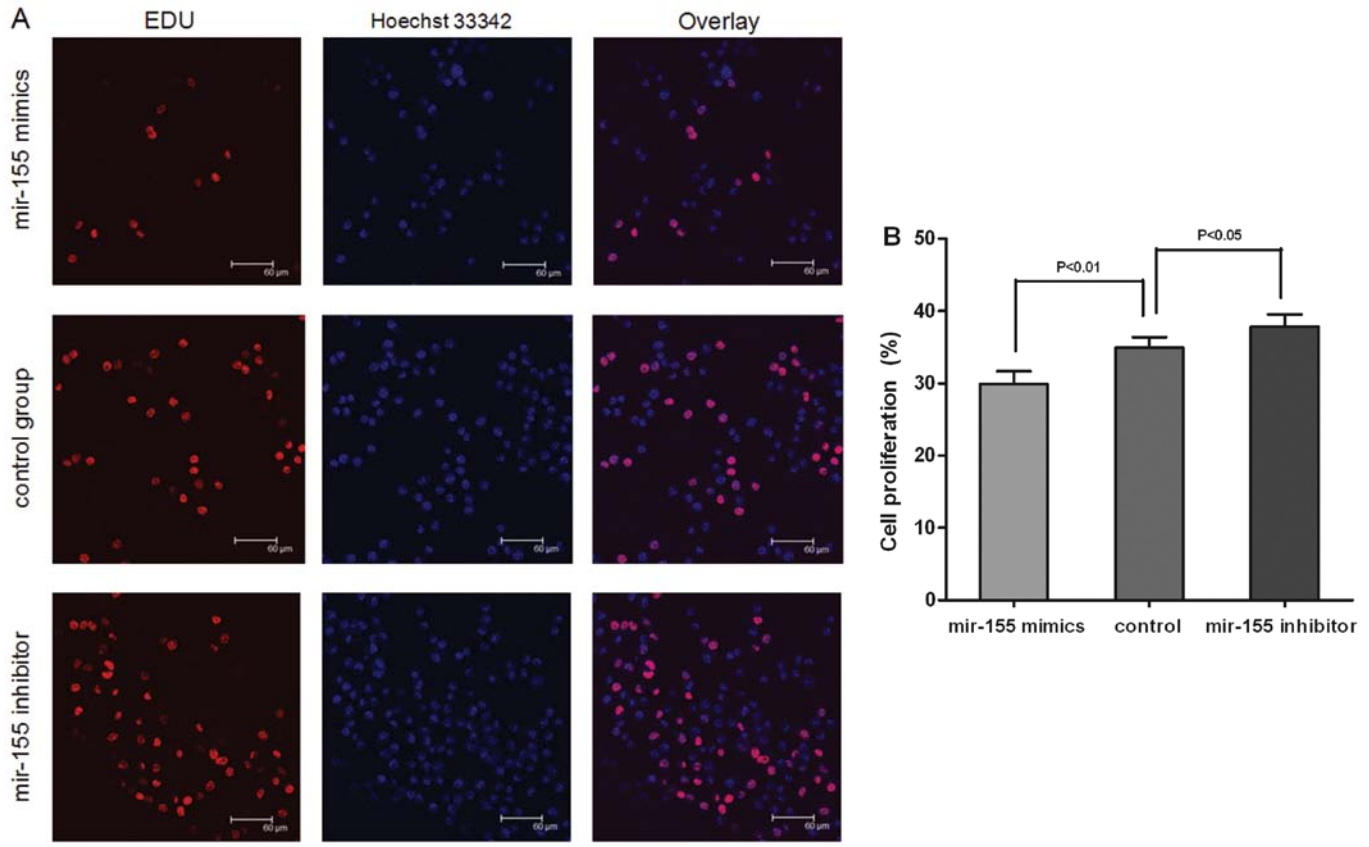

Figure 3. 5-Ethynyl-2'-deoxyuridine (EdU) is a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis only by proliferating cells. After incorporation, a fluorescent molecule was added that reacted specifically with EdU, making possible fluorescent visualization of proliferating cells (25-27). (A) Proliferating SGC-7901 cells were labeled with EdU. The reaction revealed EdU staining (red). Cell nuclei were stained with Hoechst 33342 (blue). The images are representative of the results obtained. (B) The percentage of proliferating cells was quantified by Image-Pro Plus (IPP) 6.0 software.

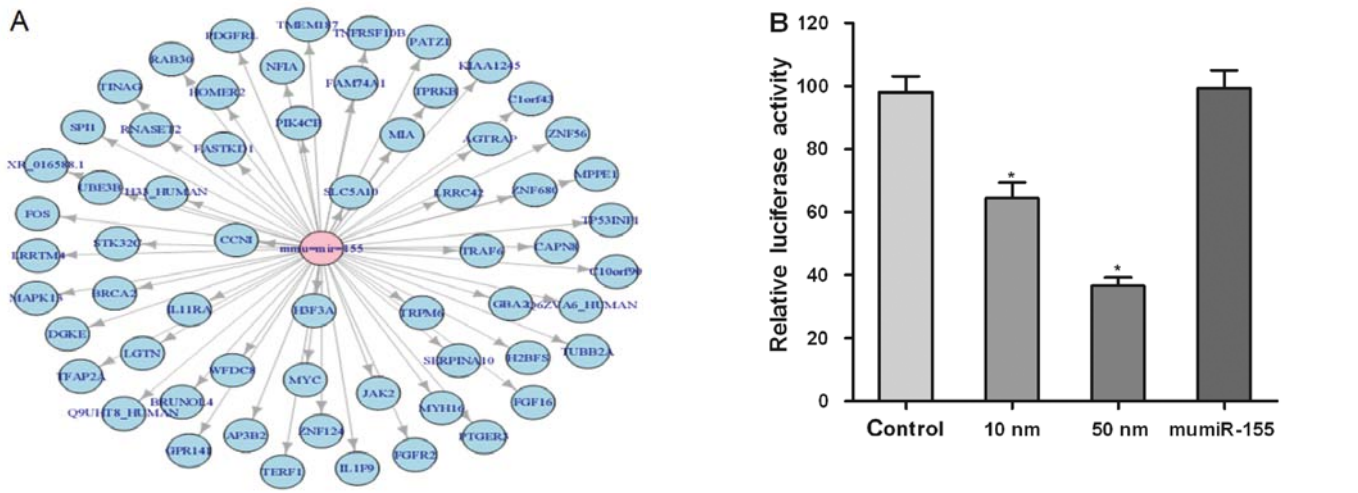

Figure 4. Targets of miR-155. (A) Network of miR-155 according to the data of the Sanger base. (B) The 3'-UTR of the cancer protein coding genes enable cancer miR-155 regulation. The data present the relative repression of firefly luciferase expression standardized to a transfection control, *Renilla* luciferase. miR-155 specifically inhibits luciferase reporter activity. HEK293 cells were cotransfected with pMIR/PRLR/5'→3', and 10, 50 nM of a given miR-155 mimic, mumiR-155. Twenty-four hours after transfection luciferase activities were measured. F-luc activity was normalized to r-luc expression and the mean activities ± SE from 5 independent experiments are shown. *P<0.01 vs. HEK293 cells transfected with miR-control mimic.

cotransfected with the pMIR/2356/5'3' construct and miR-155 (50 nM final concentration; Fig. 4B). The experiment indicated that miR-155 may decrease c-myc expression by inhibiting the translation process.

Endogenous c-myc is suppressed by miR-155. miR-155 decreases c-myc expression in human gastric cancer SGC-7901 cells. SGC-7901 cells demonstrated the endogenous expression of the myc (3). We transfected miR-155 mimics (50 nm) into

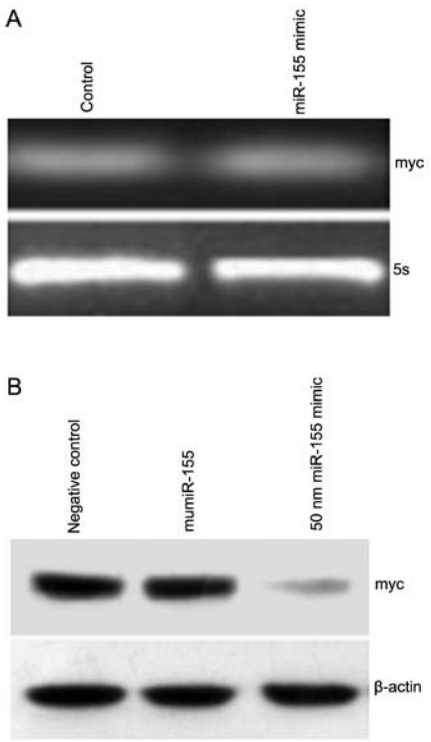

Figure 5. Post-transcriptional suppression of myc genes by the miR-155. (A) Level of myc mRNA by PCR, 5s was used as an internal standard. (B) There is a significant decrease in expression of myc protein at 50 nM of miR-155 mimic. Expression level of myc protein at 48 h after the transfection of SGC-7901 cells with miR155 mimic by western blotting. The optical density of protein band was assayed by ImageMaster VDS software.

SGC-7901 cells. As expected, myc mRNA levels were unaffected by miR-155 (Fig. 5A). In contrast, c-myc protein levels decreased substantially following treatment with miR-155, as shown by western blotting (Fig. 5B) indicating that miR-155 regulation is specific to myc.

Discussion

MicroRNAs (miRNAs) are endogenous short non-coding RNA molecules that regulate cell differentiation, proliferation and apoptosis through post-transcriptional suppression of gene expression by binding to the complementary sequence in the 3'-untranslated region (3'-UTR) of target messenger (28,29).

Gastric carcinoma develops through the accumulation of multiple genetic lesions that involve oncogenes, tumor suppressor genes and DNA mismatch repair genes (30,31). miRNAs have been proved to be closely related to the development of gastric cancer in several studies (32-34), and it is believed that they may offer a new approach for early diagnosis and individual therapy of human cancer. The relationship between gastric carcinoma genesis and the expression of miR-155 is rarely reported. In the present study, we examined, using real-time PCR, the expression of mature miR-155 in SGC-7901 cells and 52 matched pairs of gastric tumoral and non-tumoral tissues from patients. The results showed miRNA was significantly downregulated in SGC-7901 cells as well as in 32 of the 52 patients with gastric cancer. Similarly, the expression of miR-155 was reduced in solid tumors (19).

EdU has been widely used to measure the novel synthesis of DNA, and already takes the place of bromodeoxyuridine; it is simple and reproducible and may keep excellent preservation of nuclear structure due to the simple detection systems. In this study, we used EdU assay to observe the proliferation of the SGC-7901 cells transfected by mir-155 mimics or inhibitor, and the results showed that high level of mir-155 may inhibit the proliferation of SGC-7901 cells. On the contrary, the low level may lead to high proliferation. Some studies also reported that the level of mir-429 and -214 lead to similar effects of tumor cells (33,35). Meanwhile, the data showed that high level of mir-155 may reduce attachment to fibronectin and the viability of SGC-7901 cells, attachment and viability closely relate to the migration and invasion of cancer cells. These suggest that downregulation of miR-155 in solid tumor or SGC-7901 cells may play a considerable role in the development of gastric cancer through enhancing cell proliferation and cell invasion.

It is well known that the function of miRNA is decided by its target genes. Prediction of target genes found that for miR-155 there may be >900 target genes of which only a small portion has been confirmed by experiments (36). Xie *et al* reported transient ectopic expression of miR-155 was able to decrease endogenous levels of p21 by targeting SOX6 in hepatocellular carcinoma (37). Relevant targets for miR-155 can be dominant (oncogene) cancer genes, such as myc, FGFR, according to the Sanger miRbase (Fig. 4A). c-myc is overexpressed in various types of human cancers and a previous study (38) showed c-myc directly or indirectly influences cancer cell growth, proliferation and metastasis. c-myc has previously been shown to be a direct downstream target of miR-145 in non-small cell lung cancer cells (39), and miR-145 has also been shown to be an intermediate in the p53-mediated downregulation of c-myc in HCT116 cells (40) and we believe c-myc also plays an important role in the progression of gastric carcinoma. We then experimentally confirmed the predictions for myc cancer gene and was found to be positive in a luciferase assay by showing a significant reduction of protein translation in respect to the scrambled control oligoRNAs (Fig. 5B). This finding suggests a post-transcriptional mechanism for regulation of myc that could be well explained by the concomitant miR-155 downregulation we detected in gastric carcinoma for the first time. Our finding showed that high level of miR-155 may lead to the downregulation of c-myc, and serving as a pro-oncogene, c-myc is closely related to tumor cell proliferation, transformation and the induction of apoptosis, that may provide further insight into how mir-155 affected cell proliferation and viability. This experimental evidence also reinforces the hypothesis that key cancer genes are regulated by aberrant expression of miRNAs in solid cancers (41). These data add to the list of miRNAs with important cancer gene targets as previously shown by Johnson *et al* (42) (let-7, Ras interaction), O'Donnell *et al* (43) (miR-17-5p, E2F1) and Cimmino *et al* (44) (miR-16, Bcl2).

In summary, the present study identified miR-155 as a miRNA which was downregulated in solid cancer and SGC-7901 cells, and which plays a considerable role in the proliferation and viability of cancer cells. The present study indicates that miR-155 is involved in gastric carcinoma and supports its function in a recessive manner, by controlling the expression of protein-coding oncogenes. It is noteworthy that

we demonstrated for the first time that c-myc is a target of miR-155, and mir-155 directly targets the 3'-UTR of c-myc and inhibits the translation process.

Although the function and expression of miRNA regulation mechanism remains unclear, miRNA in many tumor tissues indicates that its expression in cancer diagnosis and treatment is important (45). Perhaps the tissues and fluids of miR-155 expression levels can also be used as diagnostic and prognostic tumor markers (46). However, these should be confirmed with further *in vivo* experiments and clinical analyses of larger sample sizes.

One important limitation of the present study was that only 52 patients were enrolled in the analysis of the expression level of miR-155 and the findings should be confirmed in a larger patient population. Additionally, more assays should be performed to validate the mechanism of miR-155 in tumor migration and invasion.

Acknowledgements

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