EUROPEAN JOURNAL OF CANCER 47 (2011) 2552–2559

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# miR-429 Modulates the expression of c-myc in human gastric carcinoma cells

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ARTICLE INFO

*Article history:*
Available online 16 June 2011

*Keywords:*
miRNA
miR-429
Proliferation
c-myc
Gastric cancer

ABSTRACT

*Aim:* MicroRNAs (miRNAs) are a recently discovered class of small non-coding RNAs that regulate gene expression and may contribute to the development and progression of many cancers. In this study, our goal was to investigate the regulation of miR-429 in gastric cancer and explored the mechanism/s by which it influenced pathogenesis of gastric cancer.

*Methods:* We used real-time reverse transcriptase–polymerase chain reaction to quantify the expression level of miR-429 in 52 gastric cancer tissues and their paracancerous tissues. Bioinformatics was used to predict downstream target genes of miR-429. SGC-7901 gastric cancer cells were transfected with miR-429 mimics and endogenous c-myc expression was detected by western blots. We performed functional assays using the 3′UTR of the *c-myc* gene as a miR-429 target in a luciferase reporter assay system.

*Results:* We showed that miR-429 was downregulated in human gastric carcinoma tissue and in SGC-7901 cells. Cell viability, proliferation and attachment were inhibited in miR-429-transfected cells. miR-429 significantly downregulated endogenous c-myc expression in SGC-7901 cells. Action of miR/429 on c-myc 3′UTR was confirmed. The levels of miR-429 in tumour tissue of patients with lymph node metastasis were significantly lower than in those without lymph node metastasis.

*Conclusions:* Our results suggested that miR-429 played a role in the pathogenesis of gastric carcinoma and may function as a recessive cancer gene. c-myc is an important miR-429 target gene.



## 1. Background

MicroRNAs (miRNAs), a class of small, non-coding RNAs have been shown to play a role in regulating gene expression.[1] miRNAs were originally discovered as an evolutionarily conserved class of 18–24mers which are formed by the sequential processing of primary transcripts (pri-miRNAs) by two RNase enzymes, Drosha and Dicer.[2] More than 900 human miRNAs have been identified to date. Mature miRNAs negatively regulate gene expression by binding a partially complementary or a perfectly complementary 3′-UTR region in the target gene to cause translational inhibition or mRNA degradation, respectively.[3,4] A single miRNA can target several 100 mRNAs, leading to a complex metabolic network. This property distinguishes miRNAs from small interfering RNAs which typically target a single gene.

miRNAs play a role in the aetiology and pathogenesis of various cancers by targeting a number of oncogenes or tumour suppressors genes. miR-21 regulates PTEN expression in non-small cell lung cancer[5]; miR-328 regulates hnRNP E2 expression in leukaemic blasts[6]; miR-130b regulates RUNX3 expression in gastric cancer.[7] miRNAs are differentially

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doi:10.1016/j.ejca.2011.05.021

expressed in a number of tumours. miR-17-5p, miR-20a, miR-21, miR-92 and miR-106a have been shown to be extensively involved in the pathogenesis of solid tumours, functioning as either dominant or recessive cancer genes,[8] whilst miR-143 and -145 have been reported to function as anti-oncomirs in human colorectal tumours.[9] miRNAs have also been shown to be differentially expressed in human gastric cancer. Expression of miR-768-3p,miR-139-5p, miR-378, miR-31, miR-195, miR-497 and miR-133b,miR-139-5p, miR-497 and miR-768-3p genes was significantly upregulated in non-tumourous tissues, whilst the miR-20b, miR-20a, miR-17, miR-106a, miR-18a, miR-21, miR-106b, miR-18b, miR-421, miR-340*, miR-19a and miR-658 genes were significantly upregulated in gastric cancer tissues.[10,11]

Interestingly, some miRNAs can be either upregulated or downregulated in different cancers. miR-205 is upregulated in lung, bladder and pancreatic cancers and downregulated in prostate and breast cancers.[12] The characteristic expression patterns of specific miRNAS in different cancers and their stability in serum have intensified efforts to develop these molecules as novel diagnostic biomarkers. A number of groups are also focused on exploring if miRNAs can be used as disease susceptibility markers and prognostic markers.

miR-429, a member of the miR-200 family of microRNAs, was previously shown to inhibit the expression of transcriptional repressors ZEB1/deltaEF1 and SIP1/ZEB2[13] and regulate epithelial-mesenchymal transition, which represents an important early step during metastasis. Down-regulation of miR-429 may be an important step in tumour progression.[14] miR-429 was downregulated in endometrioid adenocarcinoma,[15] whilst its upregulation in patients with serous ovarian carcinoma is correlated with survival.[16] Hu et al. reported that low-level expression of miR-200 miRNAs in this cluster predicted poor survival.[17]

In this study, our goal was to determine the role of miR-429 in gastric cancer. We evaluated the expression of miR-429 in human gastric carcinoma and normal tissue and looked at downstream targets of miR-429 in SGC-79011 gastric cancer cells in order to understand the mechanism underlying its role in the pathogenesis of gastric cancer.

## 2. Materials and methods

### 2.1. Patient samples and cell lines

All human tissue samples were obtained from surgical specimens of 52 patients with gastric carcinoma who presented between 2006 and 2007 at the Second Affiliated Hospital, Harbin Medical University, China. All tissues, including gastric carcinoma and corresponding adjacent normal tissue (paracancerous tissues), were divided into two parts, preserved in liquid nitrogen for 30 min after retrieval and histologically confirmed.

SGC-7901(a human gastric carcinoma cell line) and HEK293 (a human embryonic kidney cell line) were from the Cell Bank of Shanghai (China) and were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 in a 5% $CO_2$ incubator. Primary cells from paracancerous gastric tissue were cultured under similar conditions, for comparison studies.[18]

### 2.2. MTT assays

Logarithmically growing SGC-7901 cells were seeded in 96-well plates ($5 \times 10^3$ cells/100 μl medium/well). The culture medium was replaced after 24 h with fresh medium containing different concentrations of miR-429 mimics (0, 20, 50, 70, and 100 nM). Cells were incubated in the presence of the mimics for 24-h and then incubated for 4 h in the presence of 20 μl MTT solution (5 g/L, Sigma, Beijing, China). DMSO was added (100 μl/well) and OD values at 570 nm were recorded with the ELX 800 absorbance Microplate Reader (BioTak, Winooski, VT). miR-control (Mmu-429) was used as the control. The assay was performed three times with eight replicates.

### 2.3. Cell adhesion assays

Ninety-six-well plates were coated with fibronection or laminin (5.0 μg/well, Sigma, Beijing, China), and then blocked with 2% BSA in RPMI-1640 medium (50 μl/well) for 1 h. The plates were washed with PBS and stored until use. SGC-7901 cells were cultured in the presence of miR-429 mimics or miR-control (0, 20, 50, 70 or 100 nM) for 24 h and then resuspended in serum free medium containing 0.1% BSA. The cell concentration was adjusted to $8 \times 10^8$ cells/L. The fibronectin or laminin-coated wells were seeded with 100 μl of this cell suspension and incubated for 1 h at 37 °C. The medium was discarded and the wells were washed with 200 μl of prewarmed PBS to wash out the unattached cells. The OD values were measured as described above. The assay was performed three times with eight replicates.

### 2.5. Cell transfection and cell number counting

SGC-79011 cells were seeded in six-well plates at a concentration of $1 \times 10^5$ cells/well and transfected with an miR-429 mimic or negative control oligonucleotide (GenePharma, China) using siPORT NeoFX (Ambion, USA) according to the manufacturer's instructions. Cells were harvested 12–24 h after transfection for RNA extraction, or after 3 days for protein extraction. The cell viability was obtained by CASY measurement (CASY-1: Schärfe System, Reutlingen, Germany) according to the manufacturer's instructions.

### 2.6. Quantitative reverse transcription–PCR

Total RNA was isolated from tissue or cell cultures, using TRIzol (Invitrogen, USA) according to manufacturer's instructions. Real-time quantitative PCR analysis was performed using standard protocols on an Applied Biosystem's 7500 HT Sequence Detection System. Mature miR-429 expression was assessed using a mirVana™ qRT-PCR miRNA Detection Kit (Ambion, USA), the miR-429 primer set (GenePharma, Shanghai, China) and the 5s RNA primer set (Ambion, USA) according to the manufacturer's instructions. PCR was performed using the following conditions: one cycle of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. All reactions were conducted in triplicate and controls were performed with (1) no template and (2) no reverse transcription for each gene. The cycle number at which the reac-

tion crossed an arbitrarily placed threshold (CT) was determined for each gene, and the relative amount of each miRNA to 5sRNA was calculated as $2^{-\Delta CT}$, where $\Delta CT = (CT_{miRNA} - CT_{5s})$.[19]

### 2.7. Target (c-myc) validation with luciferase reporter system

We used the pMIR-REPORT™ miRNA Expression Reporter Vector System (Ambion, USA), which has been optimised for cloning miRNA targets and evaluation of miRNA-mediated regulation of target genes. A full-length c-myc 3′-UTR was designed based on c-myc 3′-UTR sequence (NM-002467) and synthesised by Sango Biotech (Shanghai, China). The fragments were inserted downstream of the luciferase gene in the pMIR-REPORT™-luciferase plasmid as detailed elsewhere.[20] HEK293 cells, growing in 12-well plates were cotransfected with 0.4 μg of the reporter vector and the pMIR-REPORT™-β gal control plasmid, which was used to normalise transfection efficiency. Prior to transfection, cells were synchronised by serum-starvation for 12 h. miR-429 oligonucleotides or scrambled oligonucleotides (mumiR-429) were added to each well (10 or 50 nM/well). Cells were harvested for luciferase assays 48 h after transfection. Reporter gene assays were performed in triplicate using Luciferase Assay Kits (Promega, USA). The experiment was repeated thrice.

### 2.8. Western Blot

Samples were lysed in buffer containing 1% NP-40, 0.1 M Tris (pH 8.0), 0.15 M NaCl, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Twenty micrograms of total protein was separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane with a semidry electrophoresis transfer apparatus (Bio-Rad). After blocking with 5% non-fat dry milk in TBS + 0.1% Tween-20 (TBST), blots were incubated overnight at 4 °C with primary antibodies (anti-c-Myc and β-actin, 1:1000 dilution). Secondary antibodies conjugated to horseradish peroxidase were diluted 1:5000 and blots were incubated for 1 h at room temperature. After washes with TBST, chemiluminescence was detected using standard enhanced chemiluminescence and Kodak BioMax XAR film dried, and scanned on a Typhoon PhosphorImager (GE Healthcare) for fluorescent blots. Quantification of fluorescent signal was performed using ImageQuant 5.1 software (Molecular Dynamics).

### 2.9. Statistical analysis

For the 52 patients with gastric cancer, age was expressed as mean ± standard deviation (SD), whilst gender was expressed as counts and percentages. Other continuous variables were presented by median and inter-quartile range in the tables or corresponding Box plots. The expression levels of miR-429 in the 52 gastric cancer samples and adjacent non-tumour tissues were compared using a paired *t*-test. Continuous variables were compared by independent two-sample *t*-test for two groups, or one-way ANOVA with Bonferroni corrections for three or more than three groups. The group effect on the repeat measurements of over-expressing miR-429 cell counts were tested with linear mixed model. The correlations between miR-429 expression and patient's characteristics were performed by Mann–Whitney test and Kruskal–Wallis test for the category data with two levels and over two levels, respectively. The correlation between age and miR-429 expression was performed by the Spearman's correlation coefficient. P values of <0.05 were considered statistically significant. All analyses were performed with the SPSS 15.0 statistics software (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. miR-429 was down-regulated in gastric cancer samples and in SGC-7901 cells

Our study group comprised of 52 gastric cancer patients with 36 (69.2%) males and 16 (30.8%) females. The mean age was 59.1 ± 13.2 years. We examined the expression of mature miR-429 and 5s RNA in gastric cancer tissue and adjacent, paracancerous tissue and evaluated the significance of differential miR-429 expression by comparing Ct values in these

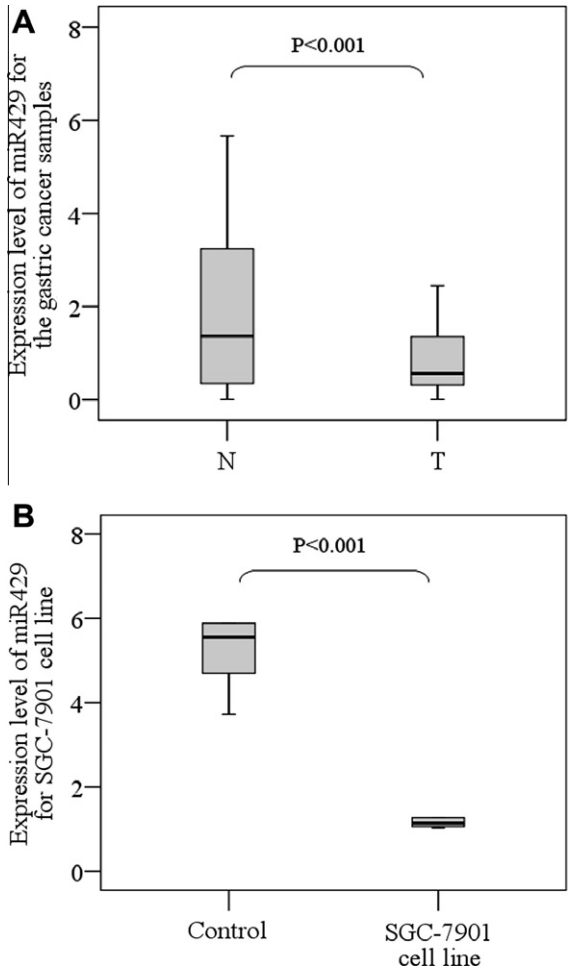


**Fig. 1 – miR-429 expression in gastric tissue and gastric cancer cells. (A) Gastric tumour tissue versus paracancerous tissue; (B) SGC-7901 cell line versus normal gastric cell.**

two tissue types. We showed that 33 patients had significantly lower levels (downregulated two-fold) of miR-429 expression in tumour tissue when compared with non-malignant adjacent gastric tissue. The average expression of miR-429 was significantly lower in gastric cancer samples (*T*), when compared with normal paracancerous tissues (*N*, Fig. 1A). We also showed significantly lower levels of miR-429 in SGC-7901 human gastric cancer cells when compared with primary cultures of non-tumour gastric cells (Fig. 1B).

### 3.2. Correlation of miR-429 expression levels with clinicopathologic parameters

The correlation of miR-429 expression levels with clinical characteristics in the 52 gastric cancer patients was analysed (Table 1). Sixteen of these patients died during the follow-up period. Most of the patients had adenocarcinomas and were in the later stages of the disease. No correlation between mir-429 levels and survival status or tumour differentiation was found. More than half the patients showed lymph node metastases. Patients with lymph node metastasis had significantly lower miR-429 expression levels in tumour tissues when compared with those without lymph node metastasis (miR-429 T/N: 0.37 versus 0.76, $p = 0.003$). Although we found no statistically significant correlation between miR-429 levels and invasion, the carcinoma with deeper invasion had lower miR-429 level. miR-429 levels were lower in advanced stage tumours compared to early stage tumours (0.46 versus 0.74, $P = 0.248$). These data suggested that miR-429 levels decreased with the progress of the gastric carcinoma.

### 3.3. Functional role of miR-429 in SGC-7901 cells

We evaluated the functional role of miR-429 in gastric carcinoma cells by measuring cell viability and proliferation in SGC-7901 cells which were transfected with the miR-429 mimic or miR-control oligonucleotide (Fig. 2A). CASY measurement showed a significant decrease in the viability of miR-429 transfected SGC-7901 cells when compared with control cells which were transfected with scrambled nucleotide (mu-miR-429) (Fig. 2B). MTT assays demonstrated a dose-dependent inhibition of cell proliferation in miR-429-treated SGC-7901 cells. Cells treated with 50 nM or higher concentrations of miR-429 showed significantly lower rates of proliferation when compared with control miRNA-treated cells (Fig. 2C). The proliferation of cells treated with 100 nM miR-429 was lower than those treated with 20 nM miR-429 ($p < 0.001$). We also showed a significant inhibition of cell attachment on laminin or fibronection in the presence of miR-429 mimics (Fig. 2D), but not control miRNA (data not shown). There

**Table 1 – Correlation between miR-429 expression and clinical characteristics.**

| | N (%) | miR-429 (T/N)[a] | P-value |
|---|---|---|---|
| *Age (year)* | 59.2 ± 13.6 | ρ = -0.172 | 0.223 |
| *Gender* | | | |
| Female | 16 (30.8%) | 0.49 (0.22, 0.77) | 0.474 |
| Male | 36 (69.2%) | 0.47 (0.35, 1.74) | |
| *Survival status* | | | 0.369 |
| Survival | 31 (59.6%) | 0.51 (0.31, 1.64) | |
| Expired during follow-up | 16 (30.8%) | 0.40 (0.28, 0.62) | |
| Lost follow-up | 5 (9.6%) | 0.58 (0.43, 0.76) | |
| *Lymph node metastasis* | | | 0.003 |
| Yes | 29 (55.8%) | 0.37 (0.23, 0.49) | |
| No | 23 (44.2%) | 0.76 (0.48, 2.66) | |
| *Invasion* | | | 0.499 |
| Submucosa | 6 (11.5%) | 0.75 (0.58, 1.07) | |
| Muscularis | 7 (13.5%) | 0.51 (0.33, 1.70) | |
| Serosa | 39 (75.0%) | 0.43 (0.25, 1.33) | |
| *Histological classification and stage* | | | 0.868[b] |
| Adenocarcinoma | 43 (82.7%) | 0.49 (0.31, 1.33) | |
| Early | 8 (15.4%) | 0.74 (0.48, 2.62) | 0.248[c] |
| Advanced | 35 (67.3%) | 0.46 (0.25, 1.33) | |
| Non-adenocarcinoma | 9 (17.3%) | 0.41 (0.35, 0.55) | |
| *Differentiation* | | | 0.886 |
| Low | 22 (42.3%) | 0.49 (0.33, 2.02) | |
| Low to middle | 9 (17.3%) | 0.43 (0.31, 1.64) | |
| Middle | 2 (3.8%) | 0.39 (0.01, 0.78) | |
| Middle to high | 10 (19.2%) | 0.55 (0.44, 1.07) | |
| Not known | 9 (17.3%) | 0.41 (0.35, 0.55) | |

[a] The ratio of miR-429 expressed in tumour and normal paracancerous tissues.
[b] Comparison between adenocarcinoma and non-adenocarcinoma tissue.
[c] Comparison between early and advanced adenocarcinoma tissue.

was less cell attachment in the presence of 100 nM miR-429 than in the presence of 20 nM miR-429 ($P < 0.001$).

### 3.4. *Target prediction in silico*

We explored the mechanism underlying miR-429-mediated cell growth inhibition by using in silico analyses to predict its cellular targets. We used the miRanda algorithm as previously described (http://www.microrna.org/mammalian/index_new.html)[21] and Targetscan (http://www.targetscan.org/).[22] The first prediction of targets was done using the Sanger database (Version 5)[20] and TargetScan5.1. We showed that c-myc, c-myb, MYCN, CyclinA and CDK8 were some of the predicted targets of miR-429. Since c-myc is known to play a role in the progression of gastric carcinoma and to regulate cellular proliferation in gastrointestinal cancer[23–25], we elected to validate the role of miR-429 on the regulation of c-myc.

### 3.5. *miR-429 down-regulated expression of endogenous c-myc protein in SGC-7901 cells*

We used Western blots to determine the effect of MiR-429 on levels of endogenous c-myc protein in SGC-7901 cells. We showed that c-myc protein expression was significantly downregulated in extracts from miR-429-transfected SGC-7901 cells when compared with miR-429 control-transfected SGC-7901 cells (Fig. 3). However, our results did not show a significant dose-dependence between the 50 nM and 100 nM groups.

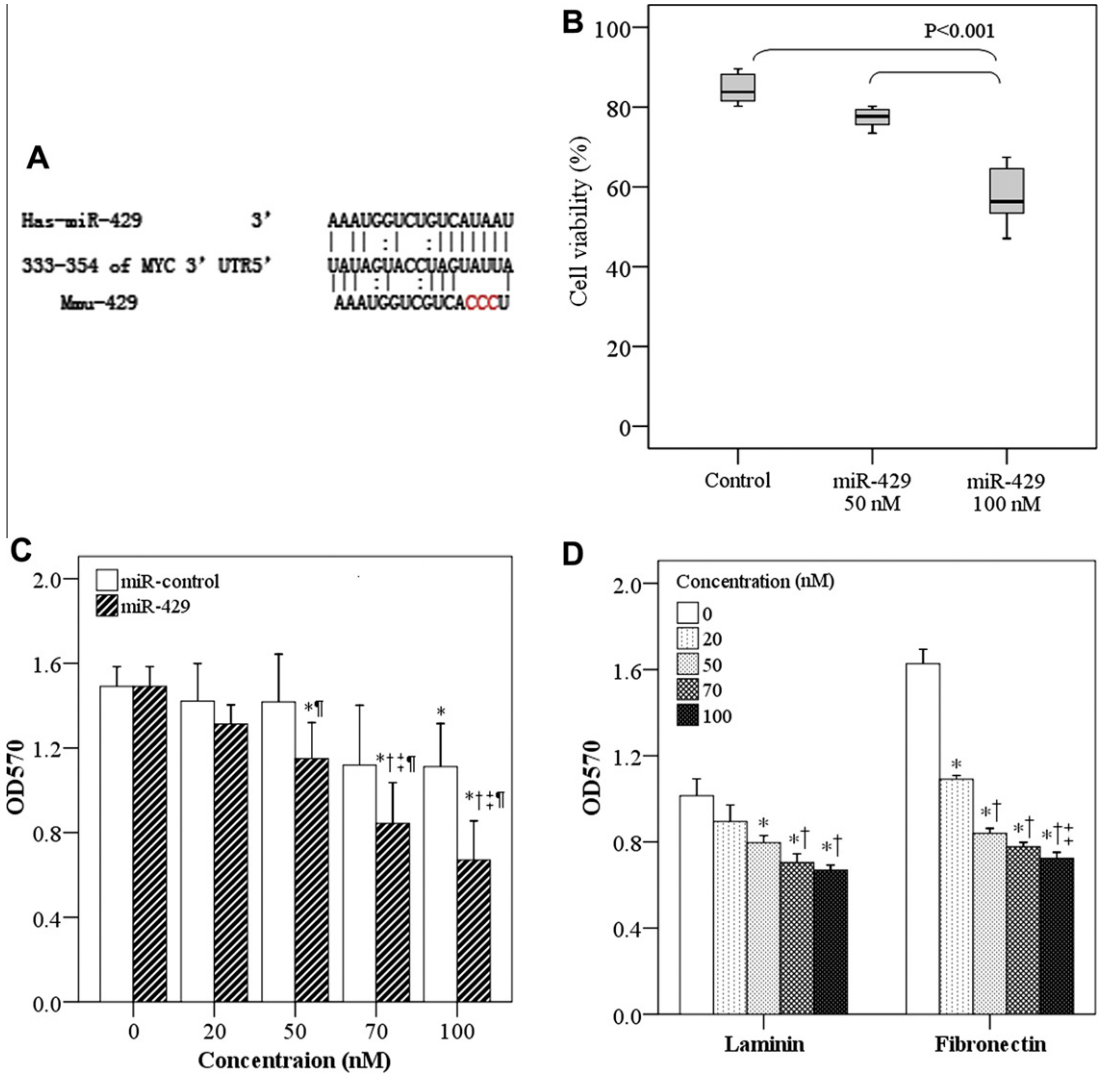


**Fig. 2 – Effect of miR-429 on cell proliferation, viability and attachment. (A) Sequences of miR-429 mimic (has-miR-429), miR-control (Mmu-429) and miR-429 target sequence (3′-UTR of Myc). (B) Viability of cells transfected with miR-control or miR-429 mimic (50 nM and 100 nM). (C) Cell proliferation in the presence of miR-429 and control. (D) adhesion assay showing the ability of cells to attach to fibronectin or laminin. *indicates a significant difference was observed as compared to the group in concentration 0 nM, †indicates a significant difference was observed as compared to the group in concentration 20 nm, ‡indicates a significant difference was observed as compared to the group in concentration 50 nm, ¶indicates a significant difference between miR-control and miR-429 groups was observed at the same concentration.**

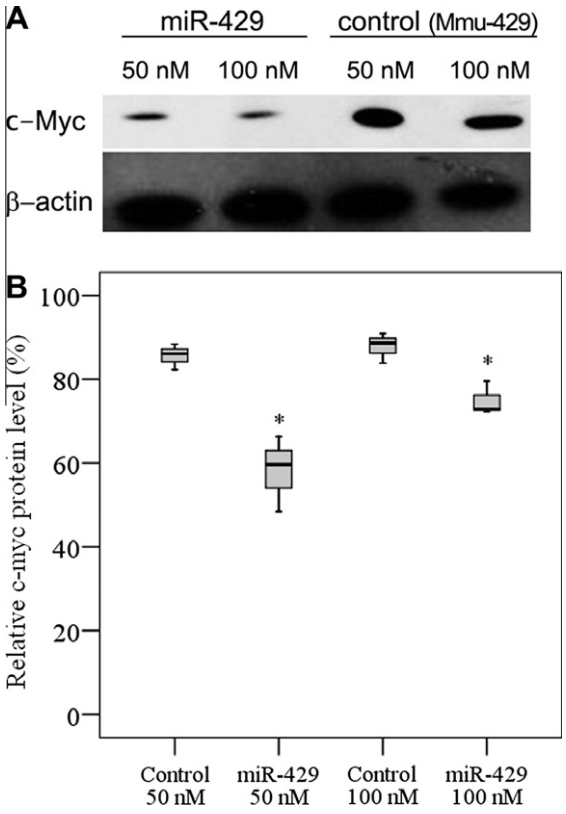


**Fig. 3 – miR-429 downregulates c-Myc expression in SGC-7901 cells. Western blot of cellular c-Myc in SGC-7901 cells transfected with 50 or 100 mM scrambled miRNA (mumiR-429) or miR-429 mimic (A). Densitometric analysis showed a significant decrease in expression of c-myc in miR-429-transfected cells ($p < 0.05$) at 50 and 100 nM (B). However, this difference was not significant after the Bonferroni adjustment.**

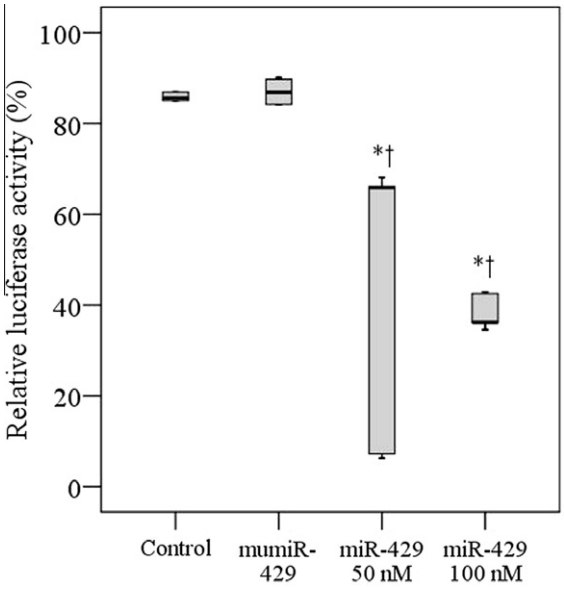


**Fig. 4 – miR-429-Mediated downregulation of c-myc validated using the miRNA Expression Reporter System.**

### 3.6. *c-myc was a target of miR-429*

In order to further validate our computational data experimentally, we cloned the full length c-myc 3′-UTR into the pMIR-REPORT luciferase plasmid (pMIR/450/5′3′). HEK293 cells were preincubated with miR-429 RNA mimics or a control RNA oligonucleotide (mumiR-429) and then transfected with pMIR/450/5′3′. We showed a significant decrease in luciferase activity in cells cotransfected with the pMIR/450/5′3′ construct and miR-429 (50, 100 nM final concentration, Fig. 4), when compared with cells that were cotransfected with pMIR/450/5′3′ and the control oligonucleotide. There was no significant difference between the two treatment groups.

## 4. Discussion

In this study, we showed for the first time that miR-429 was significantly downregulated in gastric cancer tissue when compared with adjacent normal tissue. We also showed a significant downregulation of miR-429 in SGC-7901 gastric cancer cells when compared with primary cultures of gastric non-tumour cells. Importantly, we showed that miR-429 negatively regulated c-myc expression in gastric cancer cells, suggesting a role for miR-429 dysregulation in the pathogenesis of gastric cancer.

MicroRNAs are endogenous short non-coding RNA molecules that work at the post-transcriptional level to regulate cell differentiation, proliferation, and apoptosis.

The processing of precursor miRNAs to mature miRNAs has been shown to be dysregulated in a number of cancers and this dysregulation is thought to play an important role in carcinogenesis.[3,26–28]

The let-7a, miR-21, miR-23, miR-130, miR-190, and miR-17-92 family of genes was upregulated whilst miR-122 was downregulated in human hepatocellular carcinomas.[10] Previous studies have demonstrated the role of miRNAs, notably miR-155, in the carcinogenesis of gastric tumours.[29–34] miR-451 was shown to have a tumour suppressor function in gastric cancer. miR-141 was also downregulated in a number of gastric cancer cell lines and had an inhibitory effect on cell proliferation.[35] A recent study looking at 160 pairs of non-tumour mucosa and gastric cancer showed that miR-125b, miR-199a and miR-100 were related to progression of cancer, whilst miRlet-7 g, miR-433 and miR-214 were associated with poor overall survival.[36]

In this study, we showed for the first time that miR-429 was significantly down-regulated in gastric cancer tissue as well as in SGC-7901 gastric cancer cells. miR-429 inhibited the viability and proliferation of SGC-7901 cells, suggesting that it could play a role in gastric tumourgenesis. Data from previous studies showed that miRNA106b and miRNA93 directly inhibited p21, an important CDK inhibitor[37] and facilitated progression through the G1–S phase of the cell cycle. Similarly, miR-26a was shown to directly target cyclins D1 and D2 to induce cell cycle arrest in liver cancer cells.[38] It will be interesting to explore mechanisms by which miR-429 di-

rectly or indirectly affects cell cycle progression and influences cellular transformation in gastric tumours.

We found miR-429 inhibited the proliferation of SGC-7901 cells and their attachment to fibronectin and laminin in a dose-dependent manner. The regulation of cell adhesion associates with the migration and invasion of tumour cells.[39,40] Our data suggested that miR-429 may influence cell attachment and migration. In addition, the levels of miR-429 in tumour tissue of patients with lymph node metastasis were significantly lower than in those without lymph node metastasis. These suggest that downregulation of miR-429 in tumour cells may play roles in the development of gastric cancer through enhancing cell proliferation and promoting cell migration and invasion. However, these should be confirmed with further *in vivo* experiments and clinical analyses.

Evading apoptotic pathways represents another important mechanism during cellular transformation. miRNAS play a role in regulating apoptotic pathways either directly or indirectly. miR-15 and miR-16 induce apoptosis by directly targeting the antiapoptotic protein Bcl-2[41] whilst miR-106b and miR-93 inhibit Bim expression to impair apoptosis in gastric cancer cells.[42] miRNAs have also been documented to regulate prosurvival signalling via the PI3K/Akt pathway.[4] In light of these data, we would like to address the role of miR-429 on regulation of apoptotic pathways in gastric cancer cells.

In order to understand the pathways involved in miR-429-mediated inhibition of cell proliferation, we investigated potential target genes of miR-429 in silico. Our analysis revealed *c-myc*, *c-myb*, MYCN, CyclinA, CDK8 as potential targets. Some of these target genes have been previously shown to be regulated by miRNAs. The cyclin A/cdk2 complex has been well documented to facilitate transition through the S-phase of the cell cycle and let-7b miRNA downregulated cyclin A in melanoma cells.[43] However, the implications of miR-429-mediated regulation of CDK8 are not very clear. CDK8 associates with Cyclin C to form part of the RNA polymerase II transcription machinery.[44]*c-myc*, a proto-oncogene which is over-expressed in a wide range of human cancers, has been shown to directly or indirectly influence cancer cell growth, proliferation, angiogenesis and metastasis[45] and is thought to play a role in tumour progression of gastric carcinoma.[46] Nude mice bearing SGC-7901 tumours showed a significant decrease in tumour growth when treated with recombinant antisense *c-myc* adenovirus.[46] *C-myc* has previously been shown to be a direct downstream target of miR-145 in non-small cell lung cancer cells.[47] miR-145 has also been shown to be an intermediate in the p53-mediated downregulation of *c-myc* in HCT116 cells.[48]

Using an miRNA reporter system, we showed that miR-429 specifically downregulated endogenous *c-myc* expression at the post-transcriptional level, reinforcing the hypothesis that key cancer genes are regulated by aberrant expression of miRNAs in solid cancers.[49] Our data are consistent with previous reports demonstrating the regulation of a number of important cancer targets by miRNAs, such as Ras by let-7,[50] E2F1 by miR-17-5p and Bcl-2 by miR-16.[41] Although miR-429 has been computationally predicted to act on several hundred downstream, it is very important to validate these targets by functional assays.

One important limitation of our study is that we did not do migration and invasiveness assays to validate the involvement of miR-429 in tumour migration and invasion. Only 52 patients were enrolled in the analysis of the clinic-pathological characteristics. We would like to confirm the findings in a larger patient population.

## 5. Conclusions

We found that miR-429 plays a role in the pathogenesis of gastric cancer in recessive fashion. We performed in silico analysis to predict downstream targets of miR-429 and used functional assays to confirm that miR-429 downregulated c-myc expression in gastric cancer cells.

## Authors' contributions

Tiewei Sun: experimental design, constructs, c-myc reporter gene assay, qPCRs, writing of manuscript.

Chunmei Wang: cell cultures and western blots.

Quande Wu: experimental design, discussion of experimental results, manuscript revision.

All authors drafted, read and approved the manuscript.

## Conflict of interest statement

None declared.

## Acknowledgement

This work was supported by a Grant no. (2006-10A) by Heilongjian Nature Fund.

REFERENCES

1. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* 2003;**113**(6):673–6.
2. Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell* 2004;**16**(6):861–5.
3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;**116**(2):281–97.
4. Wu WK, Lee CW, Cho CH, et al. MicroRNA dysregulation in gastric cancer: a new player enters the game. *Oncogene* 2010;**29**(43):5761–71.
5. Zhang JG, Wang JJ, Zhao F, et al. MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC). *Clin Chim Acta* 2010;**411**(11–12):846–52.
6. Eiring AM, Harb JG, Neviani P, et al. miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell* 2010;**140**(5):652–65.
7. Lai KW, Koh KX, Loh M, et al. MicroRNA-130b regulates the tumour suppressor RUNX3 in gastric cancer. *Eur J Cancer* 2010;**46**(8):1456–63.
8. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006;**103**(7):2257–61.
9. Akao Y, Nakagawa Y, Hirata I, et al. Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. *Cancer Gene Ther* 2010;**17**(6):398–408.

10. Kutay H, Bai S, Datta J, et al. Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J Cell Biochem* 2006;**99**(3):671–8.
11. Zhang L, Huang J, Yang N, et al. MicroRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci USA* 2006;**103**(24):9136–41.
12. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: Playing with fire. *FEBS Lett* 2010. [Epub ahead of print].
13. Bracken CP, Gregory PA, Kolesnikoff N, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res* 2008;**68**(19):7846–54.
14. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008;**10**(5):593–601.
15. Wu W, Lin Z, Zhuang Z, Liang X. Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. *Eur J Cancer Prev* 2009;**18**(1):50–5.
16. Nam EJ, Yoon H, Kim SW, et al. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* 2008;**14**(9):2690–5.
17. Hu X, Macdonald DM, Huettner PC, et al. A miR-200 microRNA cluster as prognostic marker in advanced ovarian cancer. *Gynecol Oncol* 2009;**114**(3):457–64.
18. Romano M, Razandi M, Sekhon S, Krause WJ, Ivey KJ. Human cell line for study of damage to gastric epithelial cells *in vitro*. *J Lab Clin Med* 1988;**111**(4):430–40.
19. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;**33**(20):e179.
20. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005;**33**(4):1290–7.
21. Enright AJ, John B, Gaul U, et al. MicroRNA targets in Drosophila. *Genome Biol* 2003;**5**(1):R1.
22. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;**120**(1):15–20.
23. Kozma L, Kiss I, Hajdu J. c-myc Amplification and cluster analysis in human gastric carcinoma. *Anticancer Res* 2001;**21**(1B):707–10 .
24. Xu AG, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins Bcl-2, p53 and C-myc in the development of gastric cancer. *World J Gastroenterol* 2001;**7**(3):403–6.
25. Ishii HH, Gobe GC, Pan W, Yoneyama J, Ebihara Y. Apoptosis and cell proliferation in the development of gastric carcinomas: associations with c-myc and p53 protein expression. *J Gastroenterol Hepatol* 2002;**17**(9):966–72.
26. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004;**101**(9):2999–3004.
27. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;**435**(7043):828–33.
28. Kluiver J, Haralambieva E, de Jong D, et al. Lack of BIC and microRNA miR-155 expression in primary cases of Burkitt lymphoma. *Genes Chromosomes Cancer* 2006;**45**(2):147–53.
29. Li Z, Zhan W, Wang Z, et al. Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. *Biochem Biophys Res Commun* 2006;**348**(1):229–37.
30. Xiao B, Liu Z, Li BS, et al. Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. *J Infect Dis* 2009;**200**(6):916–25.
31. Belair C, Darfeuille F, Staedel C. *Helicobacter pylori* and gastric cancer: possible role of microRNAs in this intimate relationship. *Clin Microbiol Infect* 2009;**15**(9):806–12.
32. Fassi Fehri L, Koch M, Belogolova E, et al. *Helicobacter pylori* induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS One* 2010;**5**(3):e9500.
33. Valeri N, Gasparini P, Fabbri M, et al. Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci USA* 2010;**107**(15):6982–7.
34. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* 2002;**99**(10):6567–72.
35. Du Y, Xu Y, Ding L, et al. Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. *J Gastroenterol* 2009;**44**(6):556–61.
36. Ueda T, Volinia S, Okumura H, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* 2010;**11**(2):136–46.
37. Kim YK, Yu J, Han TS, et al. Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Res* 2009;**37**(5):1672–81.
38. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 2009;**137**(6):1005–17.
39. Alonso DF, Tejera AM, Farias EF, et al. Inhibition of mammary tumor cell adhesion, migration, and invasion by the selective synthetic urokinase inhibitor B428. *Anticancer Res* 1998;**37**(6A):4499–504.
40. Ren J, Xiao YJ, Singh LS, et al. Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. *Cancer Res* 2006;**66**(6):3006–14.
41. Cimmino A, Calin GA, Fabbri M, et al. MiR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA* 2005;**102**(39):13944–9.
42. Petrocca F, Visone R, Onelli MR, et al. E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. *Cancer Cell* 2008;**13**(3):272–86.
43. Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. *Cell Res* 2008;**18**(5):549–57.
44. Barette C, Jariel-Encontre I, Piechaczyk M, Piette J. Human cyclin C protein is stabilized by its associated kinase cdk8, independently of its catalytic activity. *Oncogene* 2001;**20**(5):551–62.
45. Eilers M, Eisenman RN. Myc's broad reach. *Genes Dev* 2008;**22**(20):2755–66.
46. Chen JP, Lin C, Xu CP, et al. Molecular therapy with recombinant antisense c-myc adenovirus for human gastric carcinoma cells *in vitro* and *in vivo*. *J Gastroenterol Hepatol* 2001;**16**(1):22–8.
47. Chen Z, Zeng H, Guo Y, et al. miRNA-145 inhibits non-small cell lung cancer cells proliferation by targeting c-Myc. *J Exp Clin Cancer Res*;**29(1)**:151.
48. Sachdeva M, Zhu S, Wu F, et al. P53 represses c-Myc through induction of the tumor suppressor miR-145. *Proc Natl Acad Sci USA* 2009;**106**(9):3207–12.
49. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005;**120**(5):635–47.
50. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005;**435**(7043):839–43.