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RESEARCH

Down-Regulated miRNA-214 Induces a Cell Cycle G1 Arrest in Gastric Cancer Cells by Up-Regulating the PTEN Protein

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Abstract To detect the expression of miRNA-214 in human gastric cancer cell lines of BGC823, MKN45 and SGC7901, and to identify the effect of miRNA-214 on cell cycle and apoptosis of these cells. Expression of miRNA-214 in human normal gastric mucosal cell line GES-1 and human gastric cancer cell lines was detected by real-time reverse-transcription polymerase chain reaction. Antisense-miRNA-214 oligonucleotides were transfected transiently into gastric cancer cell lines to down-regulate the expression of miRNA-214. The cell cycle and apoptosis were studied by flow cytometry assay. PTEN, one of the target genes of miRNA-214 was detected by using of immunocytochemistry and Western blotting. MiRNA-214 was overexpressed in gastric cancer cell lines of BGC823, MKN45 and SGC7901 compared with normal gastric mucosal cell line GES-1. Antisense-miRNA-214 oligonucleotides significantly down-regulated the expression of miRNA-214, and increased the portion of G1-phase and decreased the portion of S-phase in BGC823 and MKN45 cells. The immunocytochemistry test and Western blotting analysis showed that the down-regulation of miRNA-214 could significantly up-regulate the expression of PTEN in BGC823 and MKN45 cells. MiRNA-214 is overexpressed in human gastric cancer cell lines of BGC823, MKN45 and SGC7901. The down-regulation of miRNA-214 could induce a G1 cell cycle arrest in them, the up-regulation of PTEN maybe one of the mechanism.

Keywords Apoptosis · Cell cycle · Gastric cancer cell line · miRNA-214 · PTEN

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Introduction

Gastric cancer (GC) is the second most common malignancy and responsible for about 10% of tumor-related death world widely [1]. Though great improvement has been achieved in the diagnosis and the treatment field, the mortality of GC still remains high. Recently, emerging evidence has shown that a class of small noncoding RNA, termed microRNA (miRNA), had been demonstrated to be associated with GC.

MiRNAs negatively regulate protein expression of specific mRNA by either translational inhibition or mRNAs degradation, functioning as oncogene or anti-oncogene in various tumors [2–5]. It was demonstrated that miR-21, let-7a microRNA, miR-106-25 cluster are related with tumorigenesis [6–8], and miR-155 and let-7 microRNA family

are involved in invasion and metastasis in GC [9, 10]. MiRNA-15b and miRNA-16 modulate multidrug resistance by targeting BCL-2 in human gastric cancer cells [11]. Stefano Volinia [12] et al. has described that miRNA-214 was upregulated in GC.

MiRNA-214 was found to be strongly implicated in such processes as cell differentiation [13], cell survival [14], apoptosis [15] and aberrantly expressed in human cancer. For example, Hua Yang [14] et al. has validated that miRNA-214 induced cell survival and cisplatin resistance through targeting the 3′-untranslated region (UTR) of the PTEN, which leads to down-regulation of PTEN protein and activation of Akt pathway in Human ovarian cancer cell lines. They examined the potential targets of miRNA-214 by searching the PicTar and miRBase database and found that 3′-UTR of human PTEN contains a putative region (nucleotides 3257–3264, NM_000314) that matches to the seed sequence of hsa-miR-214. But Cheng AM [15] et al. identified that the level of apoptosis was decreased in HeLa cells when miRNA-214 was inhibited. However, the role of miRNA-214 in GC is still unknown. Better understanding of miRNA-214 functions in GC might lead to possible improvements in the treatment of these diseases.

In this study, we evaluated the expression level of miRNA-214 in three different human gastric cancer cell lines and one human normal gastric mucosal cell line and identified that miRNA-214 might be associated with GC cell cycle arrest. Further studies discovered that PTEN was a potential functional target of miRNA-214. These findings might be helpful in identifying potential therapeutic targets for gene therapy in GC.

Materials and Methods

Design and Synthesis of Anti-miRNA Oligonucleotides

The sequences of AMOs used in the study are the exact antisense copy of the mature miRNA sequence that can be found in the miRNA Registry [16]. All the oligonucleotides of AMOs contained 2′-OMe modifications at every base. The sequences of the oligonucleotides were as follow: for AMO-miRNA-214: 5′-ACUGCCUGUCUGUGCCUGCUGU-3′; for scrambled: 5′-CAGUACUUUUGUGUAGUACAA-3′.

All oligonucleotides were chemically synthesized and purified by Shanghai GenePharma Company (Shanghai, China) and stored at −70°C before use.

Cell Culture

Human gastric cancer cell lines (BGC823, SGC7901, MKN45) and human normal gastric mucosal cell line (GES-1) were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). These cells were maintained at 37°C under an atmosphere of 5% CO_2.

Real-Time PCR Analysis of miRNA-214 Expression

Total RNAs from cells were extracted with TriZol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. 5 μl RNA was reverse-transcribed at 25°C for 10 min, 42°C for 60 min, 85°C for 5 min in a 30 μl reaction mixture using EnergicScript first-strand complementary DNA (cDNA) synthesis kit (ShineGene, Shanghai, China). Next, cDNA was quantified by real-time PCR on a FTC2000 real-time PCR system (Funglyn, Toronto, Canada) with Shine Sybr real-time qPCR kit (Shine Gene, Shanghai, China), according to the manufacturer's protocol. The total volume of the reaction mixture was 50 μl. The primers used in this study were designed with the primer express 2.0. The primer sequences of miRNA-214 and GAPDH were as follows: miRNA-214: 5′-GAGCAGGCTGGAGAA-3′(reverse); 5′-ACAGCAGGCACAGAC-3′(forward);miRNA-214 probe:5′-fam+AGGCAGTGCGCGTG-MGB-3′; GAPDH: 5′-ACCCTGTTGCTGTAGCCA-3′(reverse); GAPDH: 5′-CCACTCCTCCACCTTTGAC-3′ (forward);GAPDH probe: 5′-fam+TTGCCCTCAACGACCACTTTGTC-tamra-3′.The cycling conditions were: 93°C for 4 min, 40 cycles of 93°C for 20 s, 60°C for 30S. Quantitative real-time PCR was performed in triplicate, including no-template controls. The expression of miRNA-214 was normalized to GAPDH. The mean level of the real-time PCR experiments was calculated using $2^{-\triangle\triangle Ct}$ method with minor revision [17]. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The average Ct was calculated for both miRNA-214 and GAPDH, and $\triangle$Ct was determined as the mean of triplicate Ct for miRNA-214 minus the mean of triplicate Ct for GAPDH. The relative copy number of miRNA-214 for a sample compared with GAPDH was expressed as $2^{-\triangle Ct}$.

Oligonucleotide Transfection

Cells in exponential phase of growth were grown in six-well plate (1×10 [5] per well) for 24 h and transfected with 250 nM of AMO-miRNA-214 or scrambled oligonucleotides (as a negative control, N.C.) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in serum-free DMEM for 6 h. Transfection mixtures were prepared according to the manufacturer's instructions. After transfection, the culture media was replaced with an appropriate growth medium containing 10% FBS.

Flow Cytometry Assay

72 h after transfection, cells were trypsinized, centrifuged, washed twice with ice-cooled PBS, and then suspended in 70% chilled ethanol for more then 18 h. Cells were counted and diluted to 10^6 cells. A solution containing propidium iodide (50 μg/ml; SIGMA, St. Louis, MO, USA) and RNase A (20 μg/ml) was added to the samples and incubated for 30 min at 37°C. Then the cells were analyzed for cell-cycle profile and apoptosis rate by flow cytometry (Beckman Coulter, Brea, CA, USA).

Immunocytochemistry Assay

Gastric cancer cells were grown on glass cover slips. 72 h after transfection cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 20 min at room temperature, respectively. After treated with 0.75% H_2O_2-PBS for 30 min, cells were blocked with 5% BSA (Albumin Fraction V, Dingguo, Beijng, China) for 30 min at 37°C to suppress endogenous peroxidase activity and non-specific binding, and then incubated with mouse anti-PTEN antibody (1:50; Santa Cruz Biotechnology Company, Santa Cruz, CA, USA) at 4°C overnight, followed by rabbit-anti-mouse IgG (1:500; Golden Bridge Biological Technology Company, Beijing, China) for 30 min at 37°C. Slides were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. This was followed by dehydration and dimethyl benzene treatment, then slides were mounted with cover glasses. For non-immune staining, we used PBS instead of the primary antibody as control.

Western Blotting Analysis

Expression levels of PTEN were determined by Western blotting. Three kinds of GC cells were harvested, lyzed in lysis buffer [50 mmol/l Tris–HCl, pH 7.4, 100 mmol/l NaCl, 1 mmol/l $MgCl_2$, 2.5 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 2.5 mmol/l ethylenediamine tetraacetic acid (EDTA), 0.5% Triton X-100, 0.5% NP-40, 5 g/ml aprotinin, pepstatin A, and leupeptin]. The protein concentration was measured with the BCA protein assay (Pierce Chemical, Rockford, USA) according to the manufacturer's protocol, and was separated by 10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, USA). The membranes were blocked with buffer containing 5% low fat skim milk and 0.1% Tween-20 in Tris-buffered saline (TBST) at room temperature for 2 h and then probed with PTEN monoclonal antibody (1:100 Santa Cruz Biotechnology Company, Santa Cruz, CA, USA) and β-actin monoclonal antibody (1:1000, Santa Cruz Biotechnology Company, Santa Cruz, CA, USA) at 4° C overnight, respectively. Membranes were then washed with TBST and incubated with the second antibody (peroxidase -conjugated rabbit anti-mouse IgG[1:4000]). PTEN and β-actin protein were visualized with enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. β-actin was taken as loading control. The protein quantity was analyzed by UTHSCSA Image Tool 3.0.

The PTEN protein expression was evaluated by the ratio of PTEN/β-actin protein.

Statistical Analysis

All experiments were carried out in triplicates. Data were presented as mean±SD and analyzed with SPSS statistical analysis software (version 13.0; SPSS, Chicago, USA). The significance of difference from the control value was determined with Student's *t*-test. $P<0.05$ was considered statistically significant.

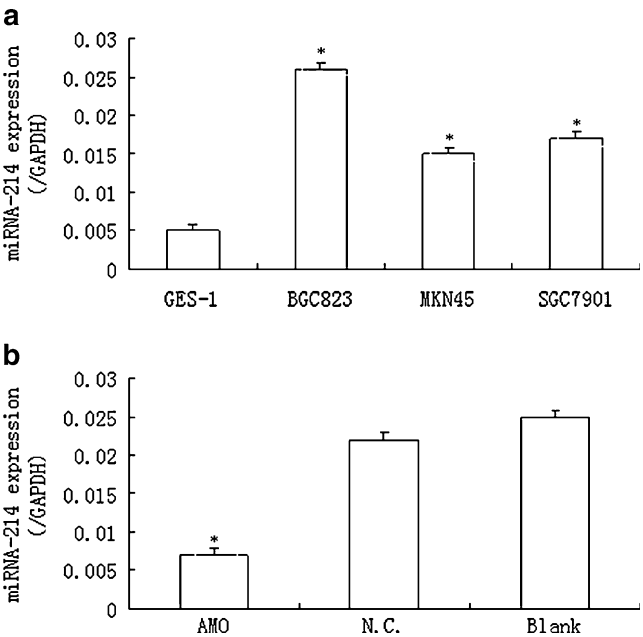

Fig. 1 **a** The expression of miRNA-214 in human normal gastric mucosal cell line GES-1 and GC cell lines by real-time PCR. $P<0.05$ when compared with GES-1. The data showed that expression of miRNA-214 was higher in GC cell lines compared with in human normal gastric mucosal cell line GES-1. **b** The expression of miRNA-214 in BGC823 AMO group and control groups by real-time PCR. $P<0.05$ when compared with N.C. and blank control. The data showed that expression of miRNA-214 was lower in AMO group compared with in control groups, indicating that AMO-miRNA-214 down-regulated the expression of miRNA-214 in BGC823 cells

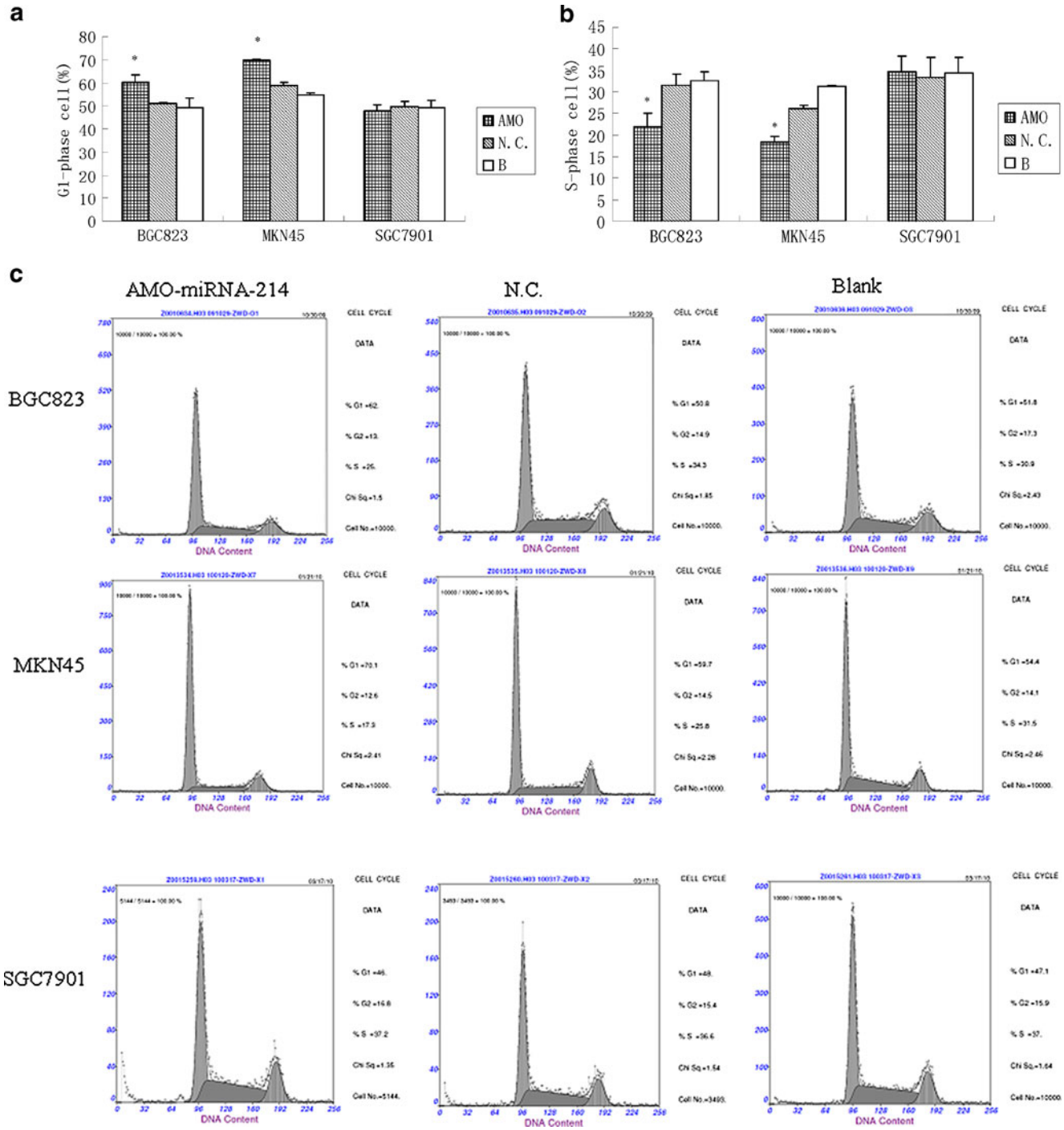

Fig. 2 **a** The percentage of G1-phase in AMO group was increased compared with control groups in BGC823 and MKN45 cells. $P<0.05$ when compared with N.C. or blank control. There was no significant difference between AMO group and control groups in SGC7901 cells. **b** The percentage of S-phase in AMO group was decreased compared with control groups in three GC cell lines. $P<0.05$ when compared with N.C. or blank control. There was no significant difference between AMO group and control groups in SGC7901 cells. **c** Effects of AMO-miRNA-214 on BGC823, MKN45 and SGC7901 cell cycle. The data indicate the percentage of live cell population in each phase of the cell cycle

Result

Real-Time PCR Determination of miRNA-214 Expression

We analyzed the expression of miRNA-214 in human normal gastric mucosal cell line GES-1 and different GC cell lines, including BGC823, MKN45 and SGC7901, by two-step quantitative RT-PCR (Fig. 1a). Thus our results showed that expression of miRNA-214 was higher in GC cell lines compared with in human normal gastric mucosal cell line GES-1. Among the three GC cell lines, BGC823 showed the highest expression level, and was chosen to the efficiency test of AMO-miRNA-214.

BGC823 cells were transfected with 250 nM AMO-miRNA-214. Cells were harvested 72 h after transfection. MiRNA-214 in AMO- miRNA-214 group and control groups was detected. As shown in Fig. 1b, the $2^{-\triangle Ct}$ value for BGC823 cells treated with AMO-miRNA-214 was 0.007 which was obviously lower than the control groups. This result indicated that AMO- miRNA-214 down-regulated the expression of miRNA-214 in BGC823 cells.

Flow Cytometric Analysis of Cell Cycle and Apoptosis of Gastric Cancer Cells Induced by AMOs

BGC823, MKN45 and SGC7901 cells were transfected with 250 nM AMO-miRNA-214. The cells were harvested 72 h after transfection and analyzed by flow cytometry. As shown in Fig. 2a, b, c, the percentage of G1-phase cells was increased in BGC823 and MKN45 cells transfected with 250 nM AMO-miRNA-214, when compared with negative control (N.C.) or blank control. The percentage of S-phase cells was decreased in BGC823 and MKN45 cells transfected with 250 nM AMO-miRNA-214, when compared

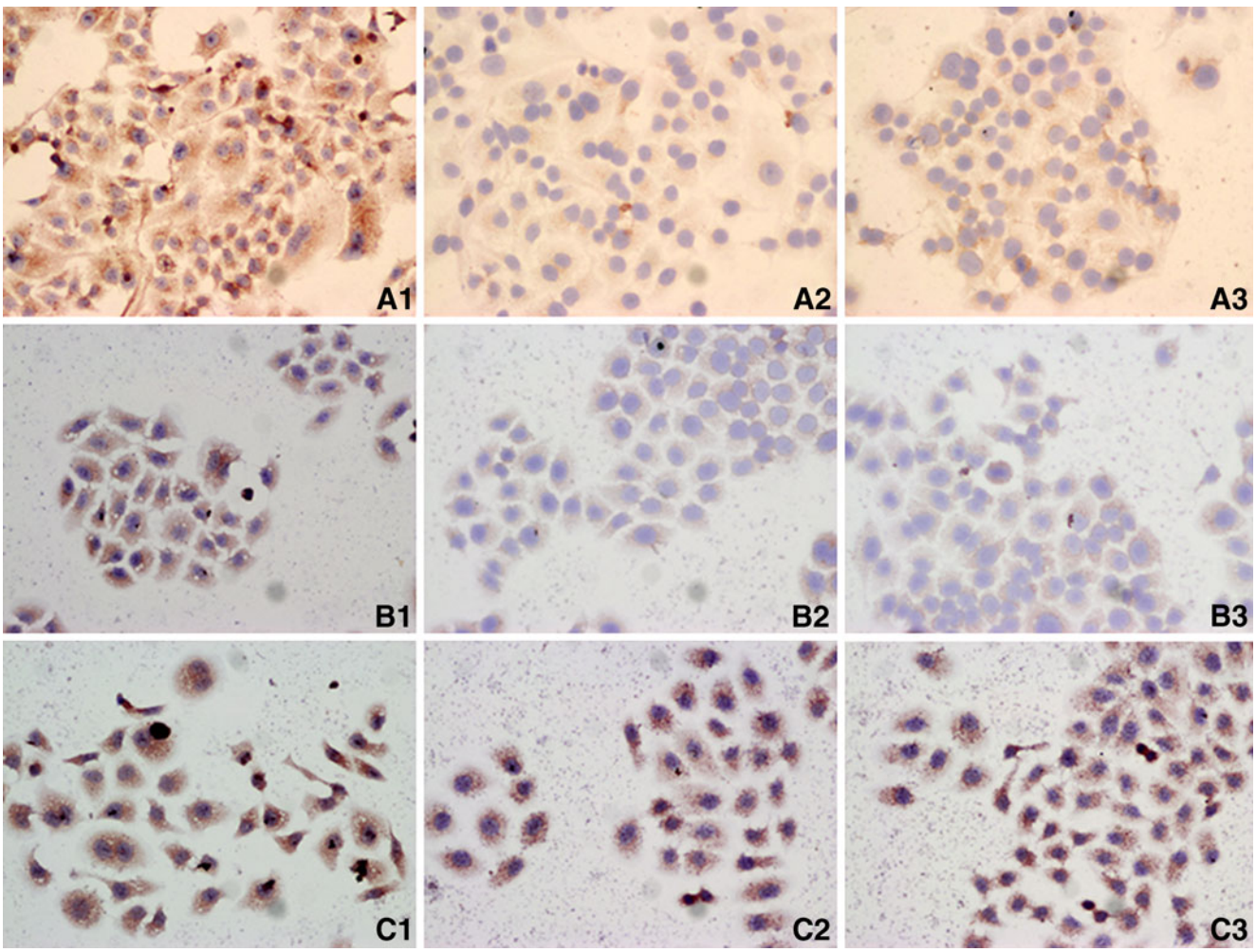

Fig. 3 Immunocytochemistry determination of PTEN protein expression in BGC823, MKN45 and SGC7901 cells. Immunocytochemistry staining indicated high constitutive levels of PTEN protein in BGC823(A1) and MKN45(B1) cells transfected with AMO-miRNA-214 compared with BGC823 and MKN45 cells transfected with scrambled oligonucleotides (A2, B2) or without transfection (A3, B3). However, cells transfected with AMO-miRNA-214(C1) did not present significantly higher expression level of PTEN protein compared with cells transfected with scrambled oligonucleotides (C2) or without transfection (C3) in SGC7901 cells

with negative control (N.C.) and blank control groups. The results indicated that AMO-miRNA-214 caused G1-phase arrest in BGC823 and MKN45 cells. However, the cell cycle distribution of SGC7901 cells in experimental group and control groups was similar. There was no significant difference in apoptosis rate between the experimental group and control groups of the three cell lines. The data indicated that AMO-miRNA-214 had no effect on apoptosis rate of BGC823, MKN45 and SGC7901 cells .All experiments were performed in triplicate.

Immunocytochemistry Determination of PTEN Protein Expression in Gastric Cancer Cells

Down-regulation or deletion of PTEN in GC has been reported previously. PTEN is also a predicted target of miRNA-214. The PTEN protein expression of three GC cell lines was evaluated by immunocytochemistry (Fig. 3). A very weak immunostaining for PTEN was observed in BGC823 and MKN45 cells transfected with scrambled oligonucleotides (Fig. 3, A2 and B2) or without transfection (Fig. 3, A3 and B3). In contrast, strong staining of PTEN protein was observed in BGC823 and MKN45 cells which were transfected with AMO-miRNA-214(Fig. 3, A1 and B1). But no significant difference was detected in the three groups of SGC7901 cells (Fig. 3, C1, C2 and C3).

Western Blotting Analysis of PTEN Protein Expression in Gastric Cancer Cells

Western blotting analysis revealed that the level of PTEN protein was markedly up-regulated in AMO groups of BGC823 and MKN45 cells ($P<0.05$)(Fig. 4). But there was no significant difference in the three groups of SGC7901 cells ($P>0.05$). These findings suggested that PTEN expression was up-regulated by AMO-miRNA- 214 in BGC823 and MKN45 cells. But AMO-miRNA-214 did not change the expression of PTEN in SGC7901 cells.

Discussion

To date, increasing data has shown the dysregulation of miRNAs in various human cancers, such as lung cancer, colorectal carcinoma, Burkitt's lymphoma and chronic lymphocytic leukemia [18–20]. MiRNAs can function as oncogene or anti-oncogene in various tumors [2–5]. Previous studies had showed that miRNA-21, miRNA-191, miRNA-223, miRNA -106b −25 cluster were up-regulated, and let-7a miRNA was down-regulated in GC tissue, indicating that these miRNAs were involved in GC tumorigenesis [7, 8, 21, 22]. Besides, miRNA-214 was also reported to be associated with various tumors [14, 23]. In this study, we firstly analyzed expression of miRNA-214 in different GC cell lines and found that expression of miRNA-214 was higher in the three GC cell lines compared with human normal gastric mucosal cell line GES-1, suggesting that miRNA-214 might involved in GC tumorigenesis process. AMO inhibition might be considered an appropriate approach to inhibit the miRNAs function and used widely.

Much evidence indicates that several miRNAs [24], including miRNA-214, could regulate the processes of cell cycle or apoptosis. MiRNA-214 might affect the generation of bipolar cells [25] and increase apoptosis of HeLa cells [15]. But Fei J [26] et al. found that AMO-miRNA-214 had no effect on cell cycle and apoptosis of A549 cell. To our knowledge, we have firstly identified that miRNA-214 might significantly modulate cell cycle of GC cell lines BGC823 and MKN45. Inhibiting miRNA-214 caused cell cycle arrest in G1-phase cells. But there was no alteration in the apoptosis rate. We speculate that there might be other dominant mechanisms regulating apoptosis in BGC823 and MKN45 cells.

Meng [27] reported that PTEN was a target of miR-21 and miR-21 modulated gemcitabine-induced apoptosis by targeting PTEN in malignant cholangiocytes. The PTEN gene is tumor suppressor and frequently deleted or mutated in human cancers. Yang H [14] et al. indicated that PTEN

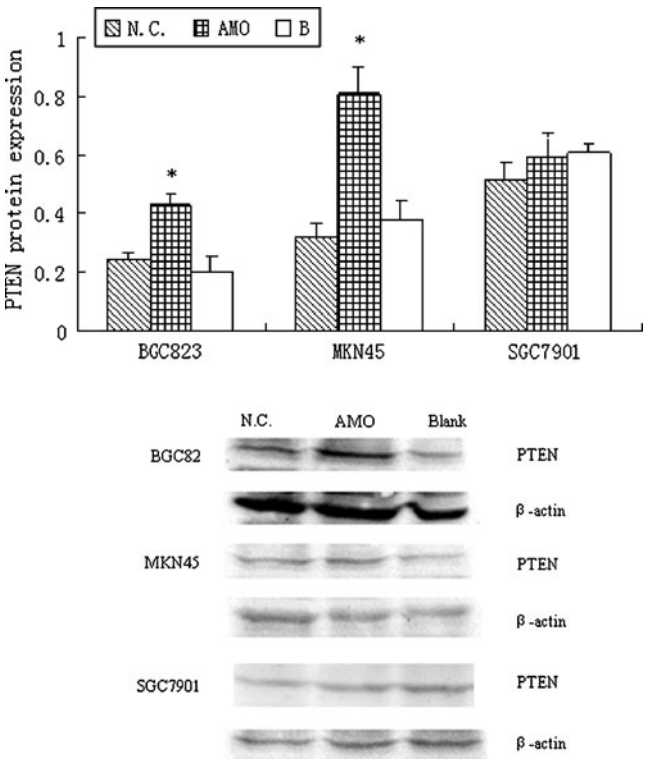

Fig. 4 Western blotting analysis of PTEN protein expression in BGC823, MKN45 and SGC7901 cells. AMO groups of BGC823 and MKN45 cells showed a higher protein expression of PTEN than control groups ($P<0.05$). But there was no significant difference in the three groups of SGC7901 cells ($P>0.05$)

was also a target of miRNA-214 and that miRNA-214 induced cell survival and cisplatin resistance primarily through targeting the PTEN/Akt pathway. Because miRNAs have different expression levels in tissues and function with tissue specificity [28], it is still unknown whether there be the same mechanism in GC cells. To study the effect of miRNA-214 on cell cycle and apoptosis in GC cell lines, we inhibited the expression of miRNA-214 with AMO-miRNA-214. The immunocytochemistry and Western blotting result showed that PTEN was up-regulated, indicating AMO-miRNA-214 might induced G1-phase arrest by regulating PTEN.

Expression loss of PTEN in mRNA level or protein level is detected frequently in GC [29]. Up-regulation of PTEN might inhibit the growth of the tumor and increase sensitivity to anticancer drugs in GC [30, 31]. Therefore, miRNA-214 and PTEN regulation might have potential therapeutic applications in the future.

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