British Journal of Cancer (2010) 103, 567 – 574

www.bjcancer.com

hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations

F Wang[1,4], X Xue[1,4], J Wei[2], Y An[1], J Yao[1,3], H Cai[1], J Wu[2], C Dai[2], Z Qian[2], Z Xu[2] and Y Miao*[,1,2]

[1]Laboratory of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210009, PR China; [2]Center for Pancreatic Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210009, PR China; [3]Department of General Surgery, The First Affiliated Hospital of Yangzhou University, Yangzhou 225001, PR China

BACKGROUND: Expression of ABCG2 is normally absent or low in the pancreas, but high in human pancreatic cancer cells. The mechanism by which ABCG2 is altered in human cancers remains unknown.

METHODS: We investigated ABCG2 expression in four pancreatic cancer cell lines, and used three microRNA (miRNA) target prediction programmes, and information from the existing literature to predict and identify hsa-miR-520h as an miRNA that targets ABCG2. The function of this miRNA was investigated by transient transfection of the pancreatic cancer cell line PANC-1 with oligonucleotides that mimic hsa-miR-520h.

RESULTS: Results showed that both mRNA and protein levels of ABCG2 were reduced, indicating that it was a target of hsa-miR-520h. Introduction of hsa-miR-520h mimics into PANC-1 cells also resulted in inhibition of cell migration and invasion, and reduction of side population cells. Cell proliferation, cell cycle progression and apoptosis were not affected.

CONCLUSIONS: We propose that the effects of hsa-miR-520h may be, at least in part, caused by its regulation of ABCG2. Thus, our findings provide a new insight into the function of miRNA in the regulation of ABCG2 expression in pancreatic cancer. Gene therapy using miRNA mimics may therefore be useful as a pancreatic cancer therapy.

British Journal of Cancer (2010) **103,** 567 – 574. doi:10.1038/sj.bjc.6605724 www.bjcancer.com
Published online 13 July 2010

Keywords: miRNA; ABCG2; migration; invasion; side population cells

Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries and has the lowest patient survival rate of any solid cancer (Jemal *et al*, 2009). One of the main reasons for its mortality is that pancreatic cancer cells have a high potential for invasion and metastasis. Although invasion and migration are the overwhelming causes of mortality in patients with solid tumours, our understanding of their molecular determinants is limited (Fidler, 2003; Weigelt *et al*, 2005; Gupta and Massagué, 2006). Studies on tumour invasion and metastasis have revealed a critical role for microRNAs (miRNAs), which are small, endogenous non-coding RNAs that post-transcriptionally regulate a variety of genes pivotal for invasion or metastasis (Lim *et al*, 2005; Dalmay and Edwards, 2006; Kim *et al*, 2008).

Expression of ABCG2, also known as breast cancer resistance protein, is normally absent or low in the pancreas (Doyle *et al*, 1998; Fetsch *et al*, 2006). However, recent studies show that ABCG2 is overexpressed in pancreatic cancer cells (König *et al*, 2005; Olempska *et al*, 2007; Guo *et al*, 2009), in which it alters the cellular epigenetic programming to promote cell survival. Moreover, ABCG2 expression is high in side-population cells of pancreatic and other tumour cell lines, as well as in primary tumour samples (Hirschmann-Jax *et al*, 2004; Seigel *et al*, 2005; Haraguchi *et al*, 2006; Shi *et al*, 2008; Sung *et al*, 2008; Dou *et al*, 2009; Wang *et al*, 2009; Yajima *et al*, 2009), in which it is likely to have a key role in xenobiotic protection, and acts as a marker of side-population cells. However, the mechanism by which ABCG2 becomes over-expressed in human cancers remains unknown, and relatively little is known about the mechanisms that normally regulate ABCG2 expression. Previous studies suggested that it may be regulated by DNA methylation (To *et al*, 2006; Turner *et al*, 2006), 5′-untranslated region (UTR) variation (Nakanishi *et al*, 2006), or histone modification (To *et al*, 2008a). Cytokines and growth factors (Evseenko *et al*, 2007; Yin *et al*, 2008), such as transforming growth factor-β, tumour necrosis factor- α, interleukin 1- β, and insulin-like growth factor 2, are proposed to be involved in ABCG2 regulation. The surface protein levels of ABCG2 do not correlate well with mRNA levels in pancreatic cancer cells (Guo *et al*, 2009), suggesting that protein-regulatory mechanisms, such as post-transcriptional regulation, post-translational modifications, proteolysis, or sequestration in cell compartments, may affect the protein, without changing the transcript levels. Therefore, additional investigations of ABCG2 expression mechanisms in pancreatic cancer are needed.

miRNAs regulate gene expression post-transcriptionally, by pairing with complementary nucleotide sequences in the 3′-UTR of specific target mRNAs (Pillai, 2005; Zamore and Haley, 2005). They also have an important role in regulating diverse cellular processes, including proliferation, differentiation, cell cycle regulation, and apoptosis (Liao *et al*, 2008; Wang *et al*, 2008, 2009, 2009; Bandi *et al*, 2009; Bhattacharya *et al*, 2009; Noonan *et al*, 2009; Ribas *et al*, 2009; Saito *et al*, 2009; Song *et al*, 2009; Ostenfeld *et al*, 2010; Spizzo *et al*, 2010). miRNAs can function either as tumour suppressors or as oncogenes, depending on the genes they

*Correspondence: Dr Y Miao; E-mail: miaoyi@njmu.edu.cn
[4]These authors contributed equally to this study.
Received 16 February 2010; revised 10 May 2010; accepted 13 May 2010; published online 13 July 2010

target (Si *et al*, 2007; Welch *et al*, 2007; Aguda *et al*, 2008; Grady *et al*, 2008; Agirre *et al*, 2009; Furuta *et al*, 2009; Gandellini *et al*, 2009; Hoffman *et al*, 2009; Hurst *et al*, 2009; Lee *et al*, 2009; Sachdeva *et al*, 2009; Tsang and Kwok, 2009; Wu *et al*, 2009; Aqeilan *et al*, 2010). Deletion or loss of a suppressive miRNA results in overexpression of target oncogenes, or conversely, activation or overexpression of an oncogenic miRNA results in silencing of tumour-suppressor targets. Thus, oncogenic miRNAs are usually overexpressed in tumours. For example, miR-125a, miR-10a, miR-21, miR-222, miR-125b, miR-7, miR-452, miR-10b, miR-373, and miR-520c induce cell migration and invasion when overexpressed in tumours or tumour cell lines (Ma *et al*, 2007; Asangani *et al*, 2008; Huang *et al*, 2008; Yan *et al*, 2008; Zhang *et al*, 2008; Zhu *et al*, 2008; Cowden Dahl *et al*, 2009; Qian *et al*, 2009; Veerla *et al*, 2009; Weiss *et al*, 2009). In contrast, suppressive miRNAs such as miR-193b, miR-122, miR-15a, miR-16–1, miR-200, miR-221, miR-126*, miR-335, miR-146a, and miR-29c lead to carcinogenesis, tumour growth, and invasion when downregulated (le Sage *et al*, 2007; Bonci *et al*, 2008; Korpal *et al*, 2008; Lin *et al*, 2008; Musiyenko *et al*, 2008; Sengupta *et al*, 2008; Tavazoie *et al*, 2008; Coulouarn *et al*, 2009; Li *et al*, 2009).

In this study, on the basis of previous reports (Liao *et al*, 2008; To *et al*, 2008b), we investigated whether hsa-miR-520h targets ABCG2 mRNA. We also investigated the role of hsa-miR-520h in proliferation, cell cycle progression, apoptosis, migration, and invasion in PANC-1 cells. As side-population cells of pancreatic cancer that overexpress ABCG2 have been identified as promoting carcinogenesis (Ho *et al*, 2007; Shi *et al*, 2008; Dou *et al*, 2009; Wang *et al*, 2009), we also investigated the effects of hsa-miR-520h on these cells. We determined whether the effects of hsa-miR-520h are linked to downregulation of ABCG2 expression. On the basis of our findings, we propose a model explaining how loss of hsa-miR-520h expression and subsequent activation of ABCG2 expression are critical events in the invasion and migration of human pancreatic cancer cells.

MATERIALS AND METHODS

Cell culture

Human pancreatic cancer cell lines BxPC-3, CFPAC-1, PANC-1, and SW1990 were obtained from Shanghai Cell Bank (Shanghai, China), and propagated in our laboratory by culturing in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO, USA), 2 mM glutamine, 100 $\mu g\,ml^{-1}$ penicillin, and 100 $\mu g\,ml^{-1}$ streptomycin. All cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2.

Target prediction

Three online programs, miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), and TarBase (http://diana.cslab.ece.ntua.gr/tarbase), were used in combination with previous reports, for predicting miRNAs that might target ABCG2. PANC-1 cells overexpressing ABCG2 were used for target miRNA verification.

miRNA transfection

miRNAs were designed and synthesised by Genepharma (Shanghai, China). hsa-miR-520h mimics were sense: 5′-ACAAAGUGCUUCCCUUUAGAGU-3′ and anti-sense: 5′-UCUAAAGGGAAGCACUUUGUUU-3′. Negative controls were sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ and anti-sense: 5′-ACGUGACACGUUCGGAGAATT-3′. MicroRNA transfection was performed using Lipofectamine 2000 (Invitrogen). PANC-1 cells were grown in six-well plates to 50% confluence before transfection. Total RNA and protein were extracted at 24 h post-transfection and used for quantitative real-time PCR (qRT–PCR) and western blot analysis. Transfections were the following: hsa-miR-520h mimics (miR-520h), negative control miRNA mimics (NC) and blank control culture medium (Mock).

qRT–PCR and western blot

Total RNA was extracted from cultured or transfected cells using Trizol (Invitrogen). ABCG2 mRNA was detected by qRT–PCR

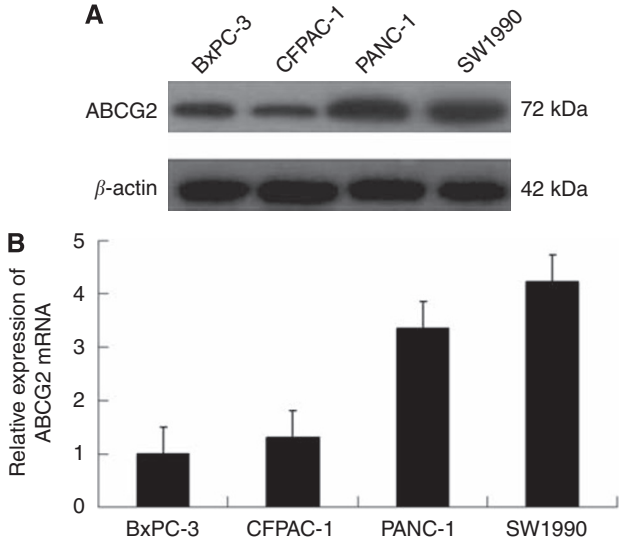

Figure 1 Expression of ABCG2 in four pancreatic cancer cell lines. (**A**) Western blot. β-Actin was used as an internal loading control. (**B**) Quantitative real-time PCR for relative expression of ABCG2 with normalisation to β-actin.

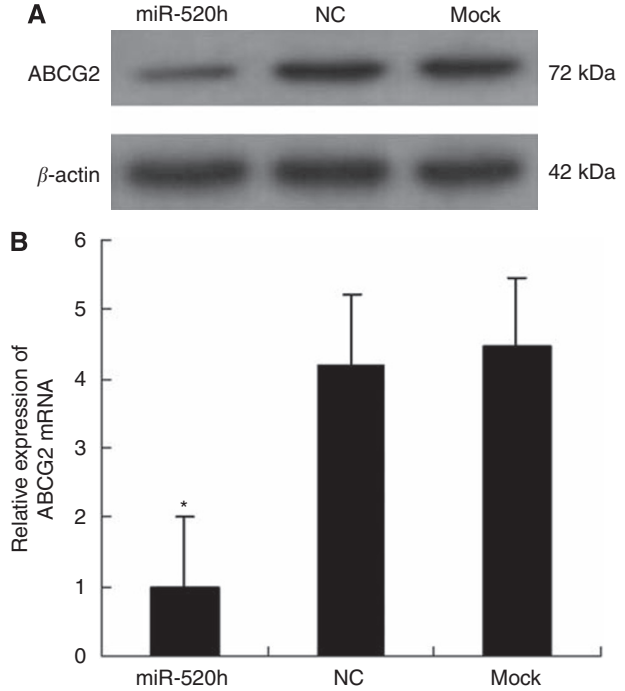

Figure 2 hsa-miR-520h targets ABCG2. (**A**) The ABCG2 protein in PANC-1 cells by western blot at 24 h post-transfection of hsa-miR-520h mimic. β-Actin was used as an internal loading control. hsa-miR-520h mimics (miR-520h), negative control miRNA mimics (NC), and blank control culture medium (mock). (**B**) ABCG2 mRNA levels were analysed by qRT–PCR at 24 h post-transfection of hsa-miR-520h mimic and normalised to β-actin. $^{*}P < 0.05$.

(Takara, Dalian, China) and normalised to β-actin. Polymerase chain reaction primers used were the following: ABCG2 sense: 5′-AATACATCAGCGGATACTACAGAG-3′, anti-sense: 5′-AGCCACC ATCATAAGGGT AAACAT-3′; β-actin sense: 5′-AGAAAATCTGGC ACCACACC-3′, anti-sense: 5′-TAGCACAGC CTGGATAGCAA-3′. Reactions were performed using an ABI PRISM 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Cultured or transfected cells were lysed using RIPA buffer with 1% PMSF. Protein concentration was estimated using a BCA kit (Keygen, Nanjing, China). Protein was resolved with 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% non-fat milk in Tris-buffered saline for 3 h and incubated overnight with primary antibodies against ABCG2 (Abcam, Cambridge, MA, USA) or β-actin (Santa Cruz, Santa Cruz, CA, USA). Membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Santa Cruz) secondary antibody and visualised with an electrochemiluminescence kit (Pierce, Rockford, IL, USA).

Cell proliferation, cell cycle, and apoptosis analysis

Cell proliferation was determined using an MTT assay. PANC-1 cells were seeded in 96-well culture plates (Costar, Cambridge) at a density of 1×10^3 cells per well, transfected with the indicated miRNA and incubated for 24 h before adding 10 μl of MTT reagent to each well and incubating at 37°C for 4 h; thereafter, the medium was replaced with 150 μl dimethyl sulphoxide (DMSO) and oscillated for 10 min. Viable cells were measured by absorbance at 490 nm wavelength using a microplate reader.

Cell cycle and apoptosis were assessed by flow cytometry (Becton Dickinson, San Jose, CA, USA). PANC-1 cells were grown and transfected as above. For cell cycle analysis, cells were collected, washed with phosphate-buffered saline (PBS) and fixed in ethanol at −20°C overnight before being collected by centrifugation, washed with PBS, and resuspended in 500 μl of PBS with 0.2% Triton X-100, 10 mM EDTA, 100 μg ml^{-1} RNase A, and 50 μg ml^{-1} propidium iodide (PI) at room temperature for 30 min. For cell apoptosis, cells were collected, washed with PBS, suspended in 100 μl 1 × binding buffer, stained with 5 μl annexin-fluorescein isothiocyanate (FITC), and 5 μl PI at room temperature for 15 min in the dark.

Cell migration and invasion analysis

Cell migration and invasion were assayed using a chamber of 6.5 mm in diameter, with 8 μm pore size (Corning, Corning, NY, USA). At 24 h post-transfection, PANC-1 cells were added to the upper chamber, which was coated with 1 mg ml^{-1} matrigel for invasion assays, and 0.6 ml of 10% FBS-DMEM was added to the lower chamber. Cells were incubated for 24 h at 37°C, and non-migrating or non-invading cells were removed with cotton swabs.

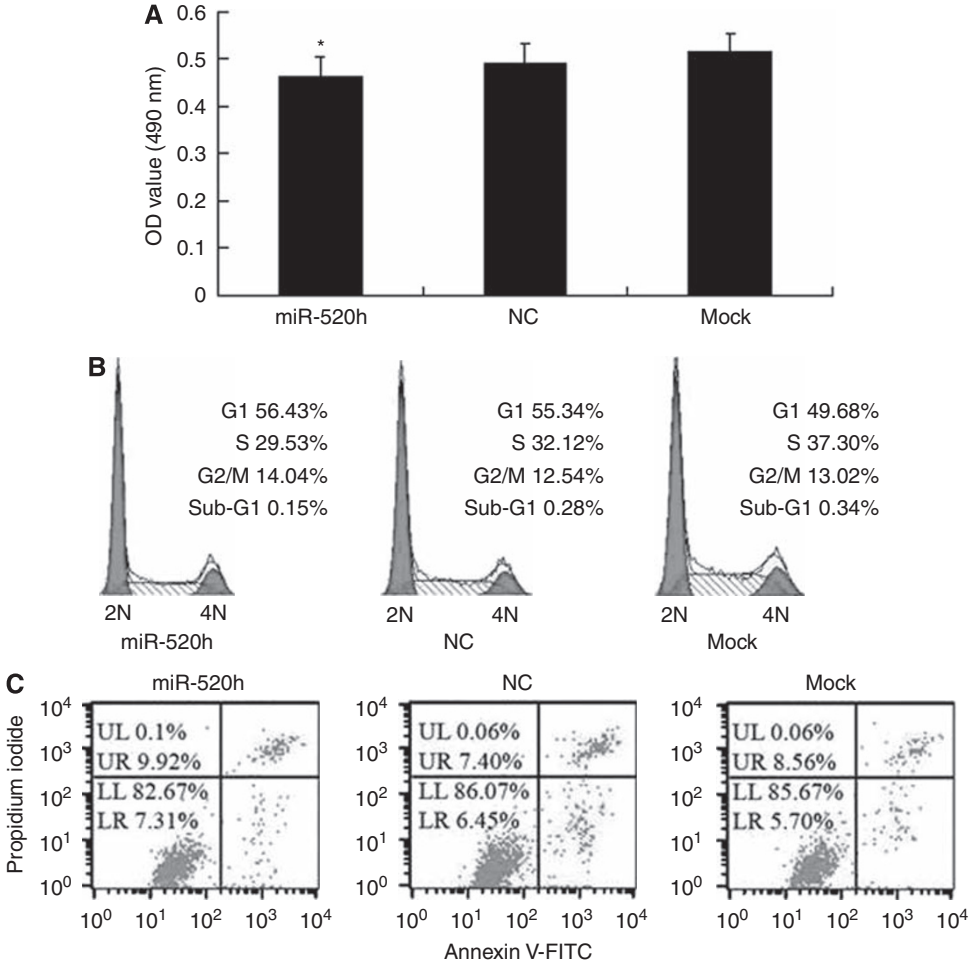

Figure 3 Effect of hsa-miR-520h on cell proliferation, cell cycle, and apoptosis of PANC-1 cells. (**A**) The proliferation ability of PANC-1 cells at 24 h post-transfection with hsa-miR-520h mimic was detected by MTT assay. The data are mean ± s.d. from three independent experiments (*$P > 0.05$, compared with controls). (**B**) Cell cycle analysis of PANC-1 cells at 24 h post-transfection was analysed by flow cytometry. 2N: cells have diploid DNA content; 4N: cells have tetraploid DNA content. (**C**) Apoptosis of PANC-1 cells at 24 h post-transfection was monitored by flow cytometry. LR is indicative of apoptosis.

Cells migrating to or invading the bottom of the membrane were stained with 0.1% crystal violet for 30 min at 37°C, and washed with PBS. Stained cells were soaked in 33% ice-cold acetic acid and oscillated for 10 min. Cell migration or invasion was assessed by absorbance at 570 nm wavelength using a microplate reader (Tecan, Shanghai, China).

Side-population cell analysis

For analysis of side-population cells, PANC-1 cells were transfected with miRNA for 24 h, then incubated with Hoechst 33342 (5 μmol ml^{-1}, Sigma) for 90 min at 37°C, either alone or in combination with 50 μmol per ml verapamil. Cells were analysed in a FACS Vantage cell sorter (Becton Dickinson) using dual-wavelength analysis (blue 425 nm, red 675 nm) after excitation with 350 nm UV light. Propidium iodide-positive cells were excluded.

Statistical analysis

Each experiment was conducted at least three times. All values were reported as mean ± s.d. Differences between experimental groups and controls were assessed by Student's *t*-test using Excel software. $P<0.05$ was considered statistically significant.

RESULTS

Pancreatic cancer cell lines expressed variable levels of ABCG2

Recent studies have revealed that ABCG2 is overexpressed in many human cancers. Therefore, we checked the expression of ABCG2 in four human malignant pancreatic cancer cell lines: BxPC-3, CFPAC-1, PANC-1, and SW1990. BxPC-3 and CFPAC-1 are low-grade cancer cell lines, and PANC-1 and SW1990 are high-grade lines. Western blot analysis showed that ABCG2 was overexpressed in PANC-1 and SW1990 cells, and underexpressed in BxPC-3 and CFPAC-1 cells (Figure 1A). These results were confirmed by qRT–PCR analysis (Figure 1B).

hsa-miR-520h targets ABCG2

Using miRNA target predicting programmes and previous reports, we hypothesised that the miRNA hsa-miR-520 h targeted ABCG2, and we chose PANC-1 cells to test our hypothesis. To verify our predicted target of hsa-miR-520h, we transfected PANC-1 cells with hsa-miR-520h mimics (miR-520h), negative control miRNA mimics (NC), and blank control culture medium (mock). Western blot analysis showed that, at 24 h post-transfection, overexpression of hsa-miR-520h resulted in a significant decrease of endogenous ABCG2 protein levels, compared with cells transformed with NC or

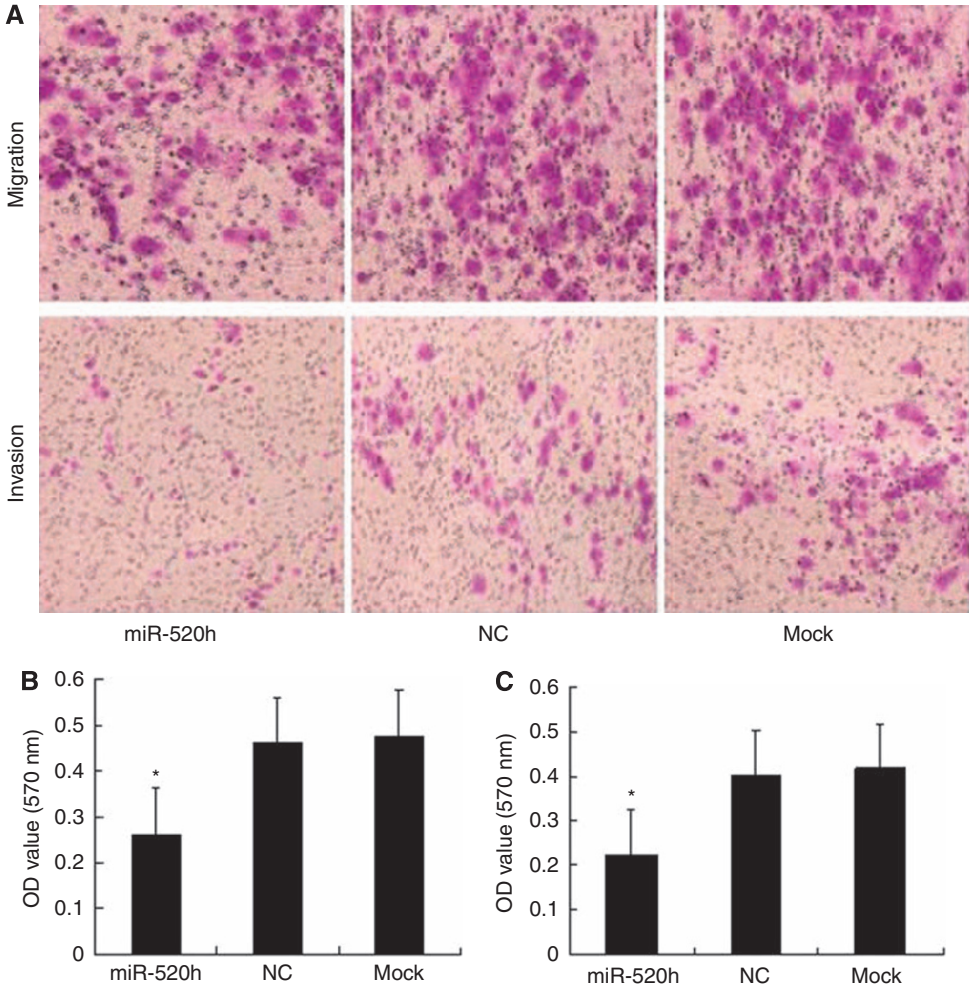

Figure 4 Overexpression of hsa-miR-520h inhibits cell migration and invasion of PANC-1 cells. PANC-1 cells were transfected with hsa-miR-520h, NC, or mock-transfected, then subjected to transwell assays, as described in Materials and Methods. After 24 h, cells were stained and measured. (**A**) Representative photographs of migrating or invading cells. (**B**, **C**) Average OD value of migration or invasion from three independent experiments ± s.d. (**B**) Cell migration. (**C**) Cell invasion. *$P<0.05$.

mock (Figure 2A). Inhibition of expression by miRNAs mediates mRNA levels; hence we used qRT–PCR to examine ABCG2's mRNA levels. Overexpression of hsa-miR-520h in PANC-1 cells led to a corresponding decrease of endogenous ABCG2 mRNA at 24 h post-transfection (Figure 2B). These observations suggested that hsa-miR-520h downregulates ABCG2 expression by inhibiting translation or causing mRNA instability. Taken together, these data indicate that hsa-miR-520h targets ABCG2.

Effects of hsa-miR-520h on proliferation, cell cycle, and apoptosis

To investigate whether hsa-miR-520h influenced cell proliferation, cell cycle, or apoptosis of PANC-1 cells by inhibiting translation of the indicated target ABCG2 mRNA, we transiently transfected PANC-1 cells with hsa-miR-520h mimics for 24 h. Ectopic expression of hsa-miR-520h had no significant inhibition on cell proliferation compared with that of control groups (Figure 3A). The cell cycle distribution of transfected cells was examined by flow cytometry, with no significant G0/G1 accumulation phenotype in PANC-1 cells transfected with hsa-miR-520h (56.43%) compared with NC (55.34%) or mock (49.68%) control-transfected cells (Figure 3B). We also examined the cell apoptosis of transfected cells by flow cytometry, and found no significant increase in apoptosis in PANC-1 cells transfected with hsa-miR-520h (7.31%) compared with NC (6.45%) or mock (5.70%) transfected cells (Figure 3C).

hsa-miR-520h inhibits migration and invasion of PANC-1 cells

To determine whether hsa-miR-520h and its target gene are associated with tumour migration and invasion, we transfected PANC-1 cells with hsa-miR-520h mimics or NC, or subjected them to mock transfection, and evaluated cell migration and invasion. As shown in Figure 4A and B, the migration ability of hsa-miR-520h mimic-transfected cells was significantly decreased by 55%, compared with that of controls. A matrigel invasion assay was performed in parallel to determine the effect of transfection with hsa-miR-520h mimics. As shown in Figure 4A and C, the invasion ability of hsa-miR-520h-transfected cells was significantly decreased by 53%, compared with that of controls. Taken together, these results suggested that hsa-miR-520h functions as a potent suppressor of PANC-1 cell migration and invasion through downregulation of ABCG2 expression.

hsa-miR-520h depletes the side population of PANC-1 cells

Previous studies suggested that ABCG2 has an important role in the identity of side-population cells. Therefore, we examined the influence of hsa-miR-520h on the population of tumour cells that exclude Hoechst 33342 dye, a strategy that is used to identify side-population cells. Flow cytometry of hsa-miR-520h-transfected PANC-1 cells showed that the side population was 1.51%, compared with 2.79% in NC-transfected and 3.06% in mock-transfected cells (Figure 5). These data indicated that

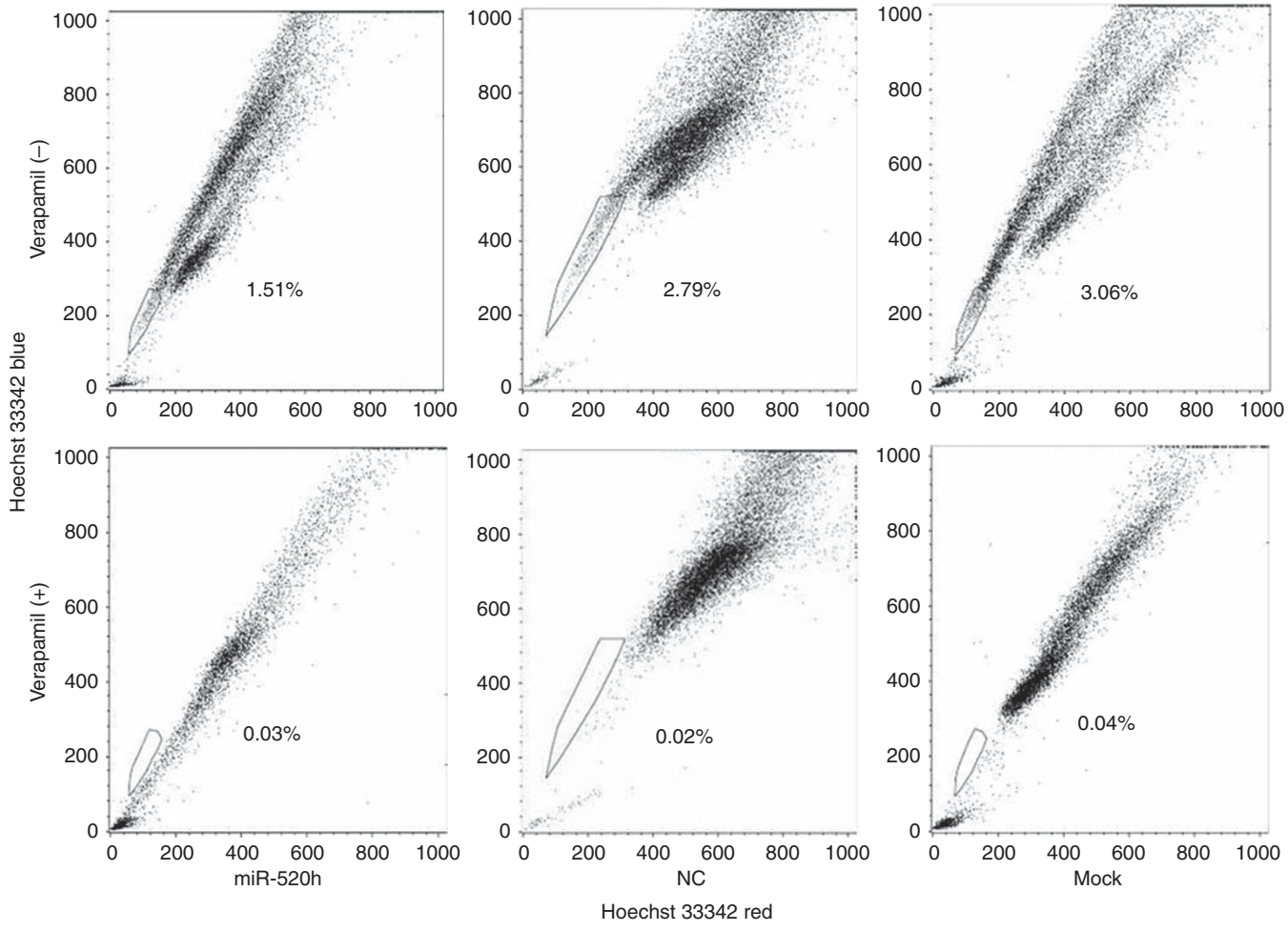

Figure 5 Overexpression of hsa-miR-520h decreases the side-population compartment of PANC-1 cells. Side-population cells of PANC-1 cells transfected with hsa-miR-520h account for 1.51% of the total number of cells, compared with 2.79% for NC-treated, or 3.06% for mock-treated, as measured by FACS analysis.

hsa-miR-520 h has a critical role in the identification of the side population, through downregulation of ABCG2 expression.

DISCUSSION

To investigate the interaction between miRNAs and their target genes in pancreatic cancer, we determined the expression levels of ABCG2 in four pancreatic cancer cell lines. Both western blot and qRT – PCR analysis showed that the cell lines expressed variable levels of ABCG2, with high expression in PANC-1 and SW1990 cells, and low expression in BxPC-3 and CFPAC-1 cells (Figure 1). We used miRNA target prediction programmes and previous studies to find an miRNA that potentially regulated ABCG2, and selected PANC-1 cells for verification using transfected miRNA mimics. In the transfected cells, ABCG2 was downregulated at both the mRNA and protein levels by the proposed target miRNA, hsa-miR-520h (Figure 2). These data suggested that ABCG2 is a target of hsa-miR-520h in pancreatic cells.

Overexpression of ABCG2 promotes migration and invasion in a variety of different tumour types (Mohan *et al*, 2006; Ho *et al*, 2007; Chiou *et al*, 2008; Okamoto *et al*, 2009). The mechanism by which ABCG2 becomes overexpressed is not entirely clear, but a possible mechanism is through miRNA regulation. MicroRNAs can function as tumour suppressors, oncogenes, or as promoters or suppressors of metastasis. We transfected hsa-miR-520h into PANC-1 cells and observed cell proliferation, cycle, apoptosis, migration, and invasion. Introduction of hsa-miR-520h resulted in a significant decrease in cell migration and invasion, but with no significant effects on cell proliferation, cell cycle distribution, or apoptosis (Figures 3 and 4). This is the first study to show that hsa-miR-520h inhibits migration and invasion in pancreatic cancer cells, through a possible mechanism of negative regulation of ABCG2 expression.

In this study, FACS analysis at 24 h after transfection of PANC-1 cells with miRNA mimics showed a remarkable decrease in the proportion of side-population cells (Figure 5). Therefore, we propose that hsa-miR-520h may have a critical role in carcinogenesis by way of affecting side-population cells by influencing ABCG2 expression. However, Patrawala *et al* (2005) reported that ABCG2-positive and -negative tumour cells have similar tumourigenicity; hence, the precise reasons why ABCG2 is associated with pancreatic cancer cells require further study.

In conclusion, we show that hsa-miR-520h has a key role not only in inhibiting migration and invasion, but also in decreasing the proportion of side-population cells of PANC-1. The possible mechanism of these effects may be through downregulation of ABCG2 expression. As no effective approaches for curing pancreatic cancer exist at present, we propose that gene therapy targeting hsa-miR-520h/ABCG2 should be investigated further as a potential alternative therapeutic strategy for pancreatic cancer.

ACKNOWLEDGEMENTS

This study was supported by a Natural Science Foundation of China grant (30972912).

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