miR-5000-3p, miR-5009-3P and miR-552: Potential microRNA biomarkers of side population cells in colon cancer

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Abstract. Colon cancer is one of the most common cancers in the world. Multidrug resistance is related to poor prognosis of advanced colon cancer. The side population plays an important role in multiple drug resistance (MDR) of colon cancer. MicroRNA biomarkers of the side population of colon cancer is still unknown. In the present study, we aimed to explore miRNA markers of side population (SP) cells of colon cancer. The side population was sorted by flow cytometry. Cell viability was measured using an MTT assay. MicroRNA profiling analysis was performed to compare microRNA expression levels in the SP cells of colon cancer with levels in the non-SP cells of colon cancer. RT-PCR was applied to verify the result obtained from the microRNA profiling analysis. miR-5000-3p, miR-5009-3P and miR-552 were all found to be upregulated in SP cells of the colon cancer cell lines HCT-15, HT-29 and LoVo. RT-PCR confirmed the result from the microRNA profiling analysis. This implied that miR-5000-3p, miR-5009-3P and miR-552 may be potential microRNA biomarkers of the side population in colon cancer, which may provide new specific targets of the side population for the reversal of MDR of colon cancer.

Introduction

Colon cancer is a common gastrointestinal cancer and is the second leading cause of cancer-related death in Western countries (1). The morbidity of colon cancer has been increasing in China during the past 20 years. Colon cancer and lung cancer as well as breast cancer are three types of cancers with the most rapidly increasing rate. The morbidity of colon cancer is ~60/100,000 individuals and its mortality is ~8/100,000 individuals (2). According to these statistics, there are almost 800,000 new patients diagnosed with colon cancer every year

in China. Moreover, almost 100,000 colon cancer patients die from colon cancer every year. Therefore, colon cancer has become a serious social health issue. Most patients with colon cancer are in its advanced stages at the time of diagnosis (3), thus surgical operation is not always a curative solution. Consequently, chemotherapy is extremely important for the treatment of colon cancer. However, the response to most forms of chemotherapy achieved to date is generally limited. Less than 50% of patients with colon cancer respond to the best regimen of combined chemotherapy. Moreover, among these patients, most of them suffer from recurrence after stopping chemotherapy for some time when relief is obtained (4). This is because colon cancer cells acquire the features of multiple drug resistance (MDR) (5).

The traditional classical mechanism of MDR in cancer cells is that cancer cells express ATP-binding cassette transporters including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), lung cancer resistance protein (LRP) and others (6). These transmembrane proteins pump chemotherapeutic drugs out of cancer cells to decrease concentrations of these drugs in cancer cells. Therefore, cancer cells are resistant to these chemotherapeutic drugs. Although MDR reversal agents targeting these ATP-binding cassette transporters including calcium antagonist such as verapamil (7), antisense RNA (8) and siRNA (9) can reverse the MDR of cancer cells *in vitro*, they do not improve the prognosis of patients when combined with antitumor drugs *in vivo* particularly in the human body.

The conception of cancer stem cells has provided a new hypothesis for the MDR of cancer in recent years. It is now currently accepted that cancer stem cells play an important role in MDR of cancer cells. Researchers are currently attempting to identify specific markers of cancer stem cells in order to exclusively kill this cell population. Various markers including CD133 (10,11) and CD44 (12) have been regarded as specific markers of cancer stem cells in cancer including colon cancer. However, CD133-negative cancer cells were found to have features of cancer stem cells in some types of cancer (13,14). Therefore, the opinion that CD133 could be regarded as a specific marker of cancer stem cells and a target of MDR of cancer cells is still controversial (15).

The side population of cancer cells is believed to have features of cancer stem cells including self-renewal and

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differentiation ability and multidrug resistance to antitumor drugs (16,17). Side population (SP) cells usually express transmembrane protein such as MDR1, BCRP1 and ABCG2. However, non-side population (non-SP) cells in many types of cancer were also reported to express BCRP1, which suggests that although these transmembrane proteins play a role in the phenotype of SP cells, they are not specific markers of SP cells (18). Behbod *et al* investigated gene markers of SP cells in breast cancer (19). However, no report exists concerning the microRNA profile comparison between SP and non-SP cells in colon cancer. The present study compared the microRNA profile of SP and non-SP cells in several colon cancer cell lines in order to explore the potential microRNA biomarkers of side population in colon cancer, which may provide new specific targets of the side population for the reversal of MDR of colon cancer.

Materials and methods

Materials. RPMI-1640 medium, Hoechst 33342, methylthiazol tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and TRIzol were purchased from Invitrogen. 5-Fluorouracil was purchased from Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). Oxaliplatin was purchased from Sanofi-Synthelabo Co. Adriamycin was purchased from Pharmacia & Upjohn Co.. miRNeasy Mini kit was purchased from Qiagen Co.. MicroRNA array analysis was performed using miRCURY™ LNA array (v.18.0) from Exiqon Co. (Vedbaek, Denmark). A reverse transcriptase kit was provided by Kangchen Bio-tech Inc. (Shanghai, China). PCR amplification was performed using Gene Amp PCR System 9700 from Applied Biosystems. Flow cytometry was performed using BD FACSAria II fluorescence-activated cell sorting system from BD Biosciences.

Cell culture. The human colon cancer cell lines, HCT-15, HT-29 and LoVo, were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences. The three colon cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified incubator containing 5% CO_2.

Side population analysis. Side population analysis was performed as described previously, with some modifications (20). Trypsinized cultured cells were washed with PBS and were resuspended at 37˚C in RPMI-1640 medium supplemented with 5% FBS. After a 10-min preincubation, the cells were labeled with Hoechst 33342 dye for 90 min at a concentration of 5 μg/ml. Cells were counterstained with 1 μg/ml propidium iodide to labeled dead cells. Next, 1×10^6 viable cells were analyzed and sorted using a BD FACSAia II fluorescence-activated cell sorting system. The Hoechst dye was excited at 355 nm and its fluorescence was measured at two wavelengths using optical filters 450 DF20 [450/20 nm band-pass filter O (Hoechst blue)] and 635LP [635 nm longpass edge filter (Hoechst red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

MTT assay. Cell proliferation assays were performed by MTT assay (21). Cells were seeded at 1×10^4/well in 96-well microtiter plates. After a 24-h incubation, an antitumor drug was added. Then cells were incubated at 37˚C in 5% CO_2 for 72 h. Then 30 μl of 5 mg/ml MTT solution was added and incubated for 4 h at 37˚C. Medium and MTT solution were discarded after a 4-h incubation. DMSO (150 μl) was added into each well to stop the reaction and shaken for 5 min. The optical density (OD) value was read on a Synergy HT multi-detection micro-plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at λ=570 nm.

MicroRNA array analysis. MicroRNA array analysis was performed by Kangchen Bio-tech, Inc. (Shanghai, China). The protocol was as follows.

RNA extraction. Total RNA was isolated using TRIzol and the miRNeasy Mini kit according to the manufacturer's instructions, which efficiently recovered all RNA species, including miRNAs. RNA quality and quantity were measured using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA integrity was determined by gel electrophoresis.

RNA labeling. After RNA isolation from the samples, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon) was used according to the manufacturer's guideline for miRNA labeling. One microgram of each sample was 3'-end-labeled with the Hy3™ fluorescent label, using T4 RNA ligase by the following procedure: RNA in 2.0 μl of water was combined with 1.0 μl of CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37˚C and was terminated by incubation for 5 min at 95˚C. Then 3.0 μl of labeling buffer, 1.5 μl of fluorescent label (Hy3™), 2.0 μl of DMSO, 2.0 μl of labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16˚C and terminated by incubation for 15 min at 65˚C.

Array hybridization. After terminating the labeling procedure, the Hy3™-labeled samples were hybridized on the miRCURY LNA array (v.18.0) according to the array manual. The total 25 μl mixture from Hy3™-labeled samples with 25 μl hybridization buffer were first denatured for 2 min at 95˚C, incubated on ice for 2 min and then hybridized to the microarray for 16-20 h at 56˚C in a 12-Bay hybridization system (Hybridization System; Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signals. Following hybridization, the slides were achieved, washed several times using wash buffer kit (Exiqon) and finally dried by centrifugation for 5 min at 400 rpm. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

Data analysis. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in all samples were chosen for calculating the normalization factor. Expression data were normalized using the median normalization. After normalization, differentially expressed miRNAs were identified through fold change filtering. To identify differentially expressed miRNAs, we performed a fold change filtering between the two samples from the experiment. The threshold we used to screen upregulated or downregulated miRNAs with a fold change ≥2.0.
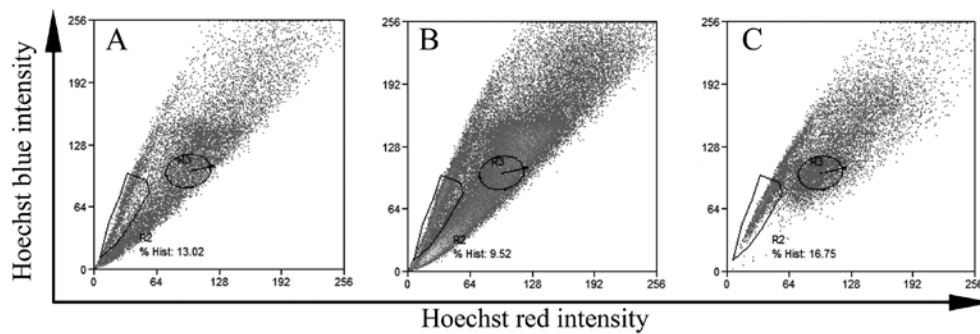
Figure 1. Side population analysis in three types of colon cancer cell lines. (A) The ratio of SP cells in the HT-29 colon cancer cell line. (B) The ratio of SP cells in the LoVo colon cancer cell line. (C) The ratio of SP cells in the HCT-15 colon cancer cell line. SP cells, side population cells.

Hierarchical clustering was performed using MEV software (v4.6, TIGR).

Verification of microRNA expression using RT-PCR. This part of the experiment was completed by Kangchen Bio-tech, Inc. The protocol in detail was as follows. Total RNAs were isolated from both SP and non-SP cells using TRIzol and the miRNeasy Mini kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 0.8 μg of total RNA by reverse transcription using MMLV reverse transcriptase (Epicentre). The primers were: RT primer 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATT GCACTGGATACGACTCCAAG-3' for miR-5000-3p; 5'-GT CGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTTTTGG-3' for miR-5009-3p; 5'-GTC GTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACT GGATACGACTTGTCTA-3' for miR-552; 5'-CGCTTC ACGAATTTGCGTGTCAT-3' for U6 as an internal control. The PCR amplification was performed in 10 μl of PCR mixture containing 2 μl of cDNA mixture, 0.5 μl of *Taq* DNA polymerase (Qiagen) and 1 μl 10 μM of the primers. The PCR mixture was initially incubated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 60 sec. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5'-GGG TCAGGACACTTCTGAA-3' and 5'-CAGTGCGTGTCGTG GAG-3' for miR-5000-3p, with an expected product size of 65 bp; 5'-GGGGGTCCTAAATCTGAAAGT-3' and 5'-GTG CGTGTCGTGGAGTCG-3' for miR-5009-3p, with an expected product size of 65 bp; 5'-GGGGGAACAGGTGACT GGT-3' and 5'-GTGCGTGTCGTGGAGTCG-3' for miR-552, with an expected product size of 64 bp; 5'-GCTTCGGCAGCAC ATATACTAAAAT-3' and 5'-CGCTTCACGAATTTGCG TGTCAT-3 for U6, with an expected product size of 89 bp. U6 was used as an internal control. The relative abundance of each microRNA was normalized by the expression level of U6 RNA, according to the formula: $\Delta\Delta Ct = (Ct_{sample} - Ct_{U6sample}) - (Ct_{control} - Ct_{U6control})$ and the estimated expression ratio was equal to $2^{\Delta\Delta Ct}$.

Results

Side population analysis. Colon cancer cells were labeled with Hoechst 33342 and then the side population of colon cancer cells were sorted by flow cytometry. As shown in Fig. 1, there

was a certain ratio of SP cells in the different colon cancer cell lines in spite of the different ratio of SP cells. The ratio of SP cells in the HCT-15, HT-29 and Lovo colon cancer cell lines was 16.75, 13.02 and 9.52%, respectively.

Cell viability of SP and non-SP cells in colon cancer cell lines treated with antitumor drugs. SP and non-SP cells sorted from the HCT-15, HT-29 and LoVo colon cancer cell lines were treated with different concentrations of 5-fluorouracil, oxaliplatin and adriamycin, respectively, for 3 days. Then cell viability was measured. As shown in Fig. 2A, the cell viability of the SP cells derived from the HCT-15 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of 5-fluorouracil after the HCT-15 colon cancer cells were treated with different concentrations of 5-fluorouracil for 3 days. IC_{50} of 5-fluorouracil for the SP cells from the HCT-15 colon cancer cell line was ~100 μg/ml. In contrast, IC_{50} of 5-fluorouracil for the non-SP cells from the HCT-15 colon cancer cell line was ~20 μg/ml. In other word, in the HCT-15 colon cancer cell line, IC_{50} of 5-fluorouracil for the SP cells was increased by ~5-fold compared to that for the non-SP cells. Cell viability of the SP cells derived from the HCT-15 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of oxaliplatin after the HCT-15 colon cancer cells were treated with different concentrations of oxaliplatin for 3 days. IC_{50} of oxaliplatin for the SP cells from the HCT-15 colon cancer cell line was ~24 μg/ml. In contrast, IC_{50} of oxaliplatin for the non-SP cells from the HCT-15 colon cancer cell line was ~6 μg/ml. In other word, in the HCT-15 colon cancer cell line, IC_{50} of oxaliplatin for the SP cells was increased by ~4-fold than that for the non-SP cells (Fig. 2B). Cell viability of the SP cells derived from the HCT-15 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of adriamycin after the HCT-15 colon cancer cells were treated with different concentrations of adriamycin for 3 days. IC_{50} of adriamycin for the SP cells from the HCT-15 colon cancer cell line was ~60 μg/ml. In conrast, IC_{50} of adriamycin for the non-SP cells from the HCT-15 colon cancer cell line was ~10 μg/ml. In other word, in the HCT-15 colon cancer cell line, IC_{50} of adriamycin for the SP cells was increased by ~6-fold than that for the non-SP cells (Fig. 2C).

Cell viability of SP cells derived from the HT-29 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of 5-fluorouracil after the HT-29
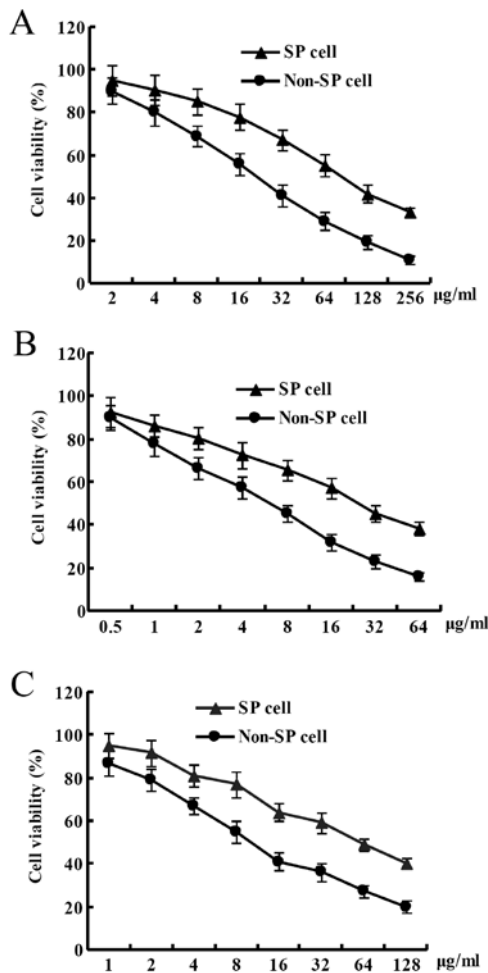
Figure 2. Cell viability of the HCT-15 colon cancer cell line treated with different concentrations of antitumor drugs. The cell viability of the HCT-15 colon cancer cell line treated with different concentrations of (A) 5-fluorouracil, (B) oxaliplatin and (C) adriamycin.
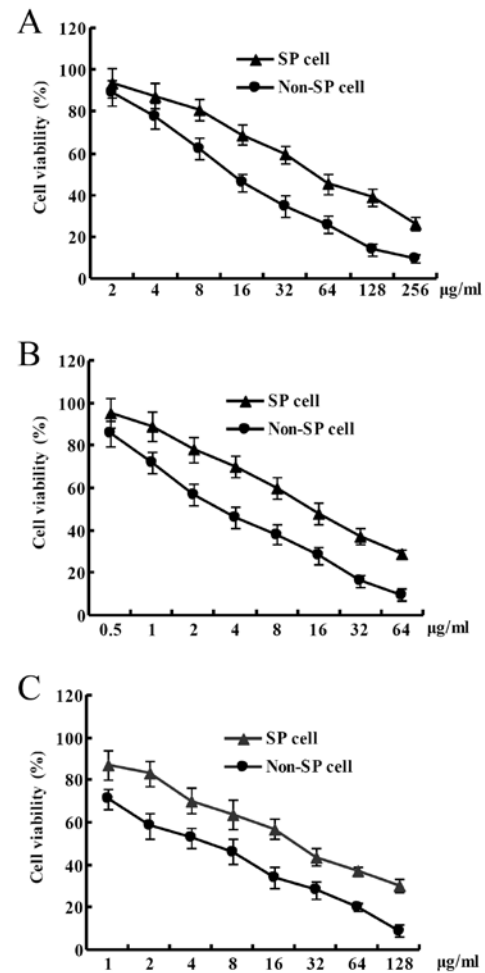

Figure 3. Cell viability of the HT-29 colon cancer cell line treated with different concentrations of antitumor drugs. Cell viability of the HT-29 colon cancer cell line treated with different concentrations of (A) 5-fluorouracil, (B) oxaliplatin and (C) adriamycin.

colon cancer cells were treated with different concentrations of 5-fluorouracil for 3 days. IC_{50} of 5-fluorouracil for the SP cells from the HT-29 colon cancer cell line was ~50 μg/ml. In contrast, IC_{50} of 5-fluorouracil for the non-SP cells from the HT-29 colon cancer cell line was ~10 μg/ml. In other word, in the HT-29 colon cancer cell line, IC_{50} of 5-fluorouracil for the SP cells was increased by ~5-fold than that for the non-SP cells (Fig. 3A). Cell viability of SP cells derived from the HT-29 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of oxaliplatin after the HT-29 colon cancer cells were treated with different concentrations of oxaliplatin for 3 days. IC_{50} of oxaliplatin for the SP cells from the HT-29 colon cancer cell line was ~15 μg/ml. In contrast, IC_{50} of oxaliplatin for the non-SP cells from the HT-29 colon cancer cell line was ~3 μg/ml. In other word, in the HT-29 colon cancer cell line, IC_{50} of oxaliplatin for the SP cells was increased by ~5-fold than that for the non-SP cells (Fig. 3B). Cell viability of the SP cells derived from the HT-29 colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of adriamycin after the HT-29 colon cancer cells were treated with different concentrations of adriamycin for 3 days. IC_{50} of adriamycin for the SP cells from the HT-29 colon cancer cell line was ~25 μg/ml.

In contrast, IC_{50} of adriamycin for the non-SP cells from the HT-29 colon cancer cell line was ~5 μg/ml. In other word, in the HT-29 colon cancer cell line, IC_{50} of adriamycin for the SP cells was increased by ~5-fold than that for the non-SP cells (Fig. 3C).

Cell viability of SP cells derived from the LoVo colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of 5-fluorouracil after the LoVo colon cancer cells were treated with different concentrations of 5-fluorouracil for 3 days. IC_{50} of 5-fluorouracil for the SP cells from the LoVo colon cancer cell line was ~25 μg/ml. In contrast, IC_{50} of 5-fluorouracil for the non-SP cells from the LoVo colon cancer cell line was ~5 μg/ml. In other word, in the LoVo colon cancer cell line, IC_{50} of 5-fluorouracil for the SP cells was increased by ~5-fold than that for the non-SP cells (Fig. 4A). Cell viability of the SP cells derived from the LoVo colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of oxaliplatin after the LoVo colon cancer cells were treated with different concentrations of oxaliplatin for 3 days. IC_{50} of oxaliplatin for the SP cells from the LoVo colon cancer cell line was ~12 μg/ml. In contrast, IC_{50} of oxaliplatin for the non-SP cells from the LoVo colon cancer cell line was ~2 μg/ml. In other word, in
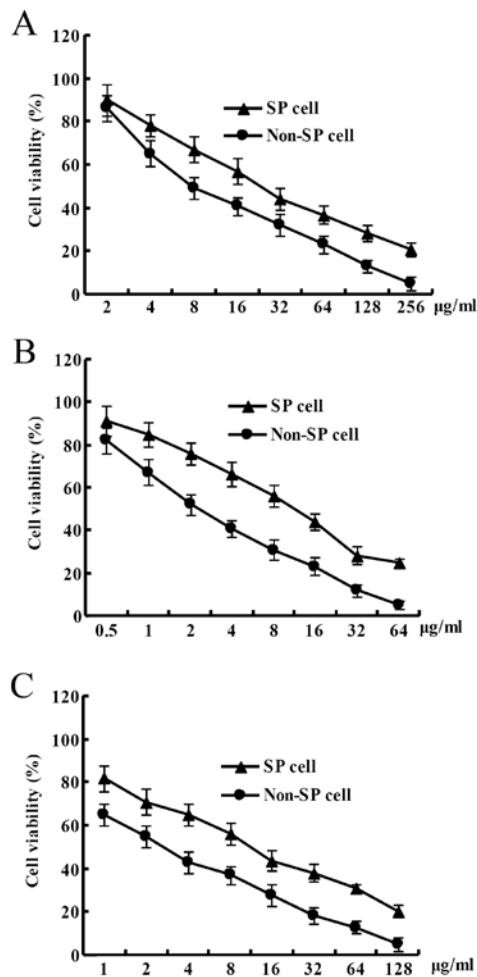
Figure 4. Cell viability of the LoVo colon cancer cell line treated with different concentrations of antitumor drugs. Cell viability of the LoVo colon cancer cell line treated with different concentrations of (A) 5-fluorouracil, (B) oxaliplatin and (C) adriamycin.
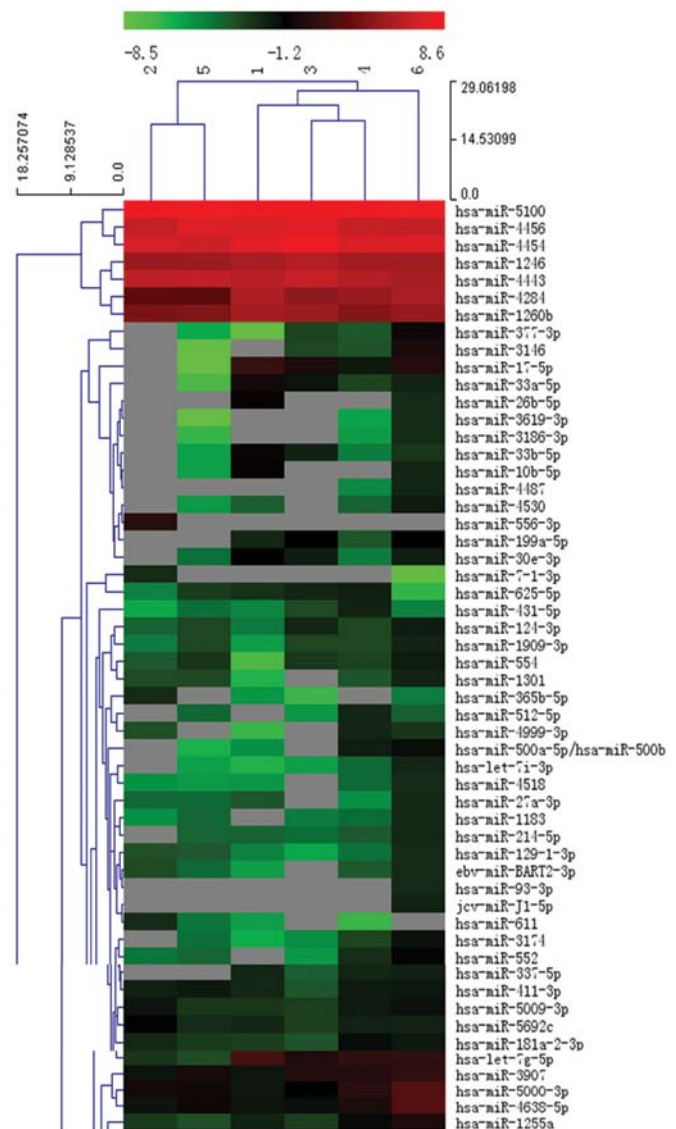

Figure 5. microRNA profiling of SP and non-SP cells in colon cancer cell lines. 1, non-SP cells of the HT-29 cell line; 2, SP cells of the HT-29 cell line; 3, non-SP cells of the LoVo cell line; 4, SP cells of the LoVo cell line; 5, non-SP cells of the HCT-15 cell line; 6, SP cells of the HCT-15 cell line. SP cells, side population cells; non-SP cells, non-side population cells.

the LoVo colon cancer cell line, IC_{50} of oxaliplatin for the SP cells was increased by ~6-fold than that for the non-SP cells (Fig. 4B). Cell viability of the SP cells derived from the LoVo colon cancer cell line was significantly higher than that of the non-SP cells at the same concentration of adriamycin after the LoVo colon cancer cells were treated with different concentrations of adriamycin for 3 days. IC_{50} of adriamycin for the SP cells from the LoVo colon cancer cell line was ~12 μg/ml. In contrast, IC_{50} of adriamycin for the non-SP cells from the LoVo colon cancer cell line was ~3 μg/ml. In other word, in the LoVo colon cancer cell line, IC_{50} of adriamycin for the SP cells was increased by ~4-fold than that for non-SP cells (Fig. 4C).

MicroRNA profiling of the side population in colon cancer cell lines. MicroRNA profiling of the SP and non-SP cells sorted from the HCT-15, HT-29 and LoVo colon cancer cell lines was carried out using miRCURY LNA array (v.18.0) microRNA chip. The differences in the microRNA profile between the SP and non-SP cells were compared in each colon cancer cell line. microRNAs with fold change >2-fold were screened. MicroRNA array analysis indicated that, in the HCT-15 colon cancer cell line, there were 106 upregulated microRNAs in the SP cells including miR-5000-3p,

miR-5009-3P, miR-552, miR-17-5p, miR-3146, miR3619-3P and others (Fig. 5); On the other hand, 52 microRNAs were downregulated including miR-133b, miR-4312, miR-4664-3p, miR-4667-3p, miR-4087-5p, miR-940 and others (data not shown). In the HT-29 colon cancer cell line, 58 microRNAs in the SP cells were upregulated including miR-5000-3p, miR-5009-3P, miR-552, miR-611, miR-365b-5p, and others (Fig. 5); However, 63 microRNAs were downregulated including miR-125b-5p, miR-30b-5p, miR-101-3p, miR-7g-5p, miR-125a-5p, miR-130b-3p, and others. (data not shown). In the LoVo colon cancer cell line, 47 microRNAs in the SP cells were upregulated including miR-5000-3p, miR-5009-3P, miR-552, miR-3915, miR-4777-5p, miR-301a-3p and others (some part of the data not shown). However, 22 microRNAs were downregulated including miR-34a-5p, miR-33b-5p, miR-30e-3p, miR-199a-5p, miR-125b-3p, miR-1275 and others (data
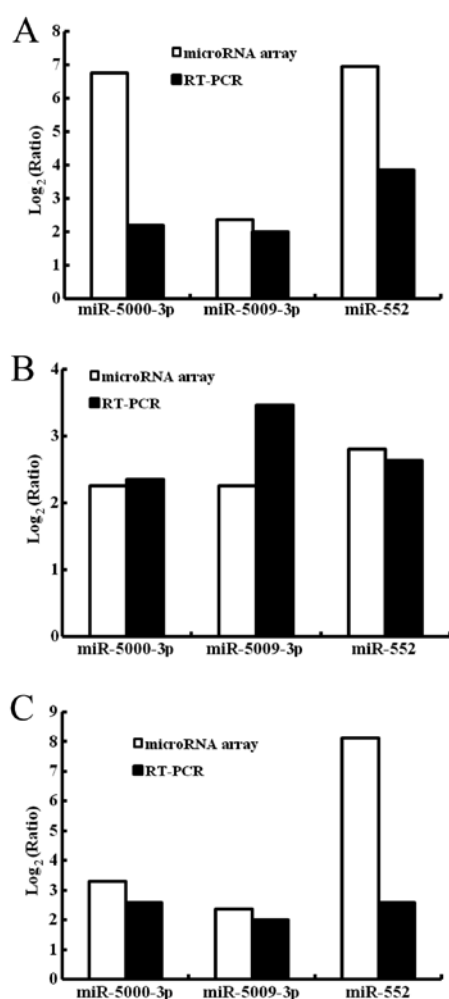
Figure 6. Comparison of the data obtained by RT-PCR and microRNA array analysis in the colon cancer cell lines. Using U6 as an internal control, miR-5000-3p, miR-5009-3P and miR-552 were assessed for expression by RT-PCR. The ratio, representing the relative value of the miRNA expression level, was expressed as a logarithm ($\log_2$). Corresponding values obtained by microRNA array analysis are presented for comparison. (A) Comparison in the HCT-15 cell line. (B) Comparison in the HT-29 cell line. (C) Comparison in the LoVo cell line.

not shown). From the above mentioned results, we found that miR-5000-3p, miR-5009-3P and miR-552 were upregulated in the SP cells of all three colon cancer cell lines (Fig. 5). However, no microRNA was found to be downregulated in SP cells of all of the three colon cancer cell lines.

Verification of three upregulated microRNAs using RT-PCR. MicroRNA profiling revealed that miR-5000-3p, miR-2009-3P and miR-552 were upregulated in SP cells of all of the three colon cancer cell lines including HCT-15, HT-29 and LoVo. To confirm and validate the results obtained from the microarray, we analyzed the expression of these three microRNAs by RT-PCR. Using U6 as an internal control, relative expression of microRNAs from the SP and non-SP cells was calculated. Then, microRNA expression detected by RT-PCR was compared with the microRNA expression obtained by the microRNA array. The ratios representing microRNA expression changes were $\log_2$-transformed in the histograms for these microRNAs. As shown in Fig. 6A, in the HCT-15 colon cancer cell line,

expression levels of miR-5000-3p, miR-5009-3P and miR-552 in the SP cells as detected by microRNA array were increased by 6.76-, 2.37- and 6.96-fold, respectively, than those of the non-SP cells while those detected by RT-PCR were increased by 2.20-, 2.01- and 3.85-fold. In the HT-29 colon cancer cell line, expression levels of miR-5000-3p, miR-5009-3P and miR-552 of the SP cells as detected by the microRNA chip were increased by 2.25-, 2.26- and 2.81-fold, respectively, than those of the non-SP cells while those detected by RT-PCR were increased by 2.35-, 3.46- and 2.63-fold (Fig. 6B). In the LoVo colon cancer cell line, expression levels of miR-5000-3p, miR-5009-3P and miR-552 of the SP cells as detected by the microRNA array were increased by 3.28-, 2.35- and 8.13-fold, respectively, than those of the non-SP cells while those detected by RT-PCR were increased by 2.57-, 2.00- and 2.59-fold (Fig. 6C). MicroRNAs including miR-5000-3p, miR-5009-3P and miR-552 detected by the microRNA chip were upregulated in SP cells of all of the three colon cancer cell lines. So were microRNAs obtained by RT-PCR. Therefore, the results obtained by RT-PCR verified those obtained by the microRNA chip. This implies that the data obtained from the microRNA array analysis were reliable.

Discussion

In the present study, we successfully isolated SP cells in colon cancer cell lines using Hoechst 33342 staining. SP cells were first described by Goodell *et al* (20) and SP cells of several types of malignancies were successfully isolated in subsequent studies (22-27). Haraguchi *et al* (28) isolated SP cells from gastrointestinal cancer cell lines. Although they reported the gene expression profiles and resistance to chemotherapeutic agents of SP cells derived from the liver cancer cell line Huh7, they did not report the microRNA profiles of SP cells. Schetter *et al* (29) and Callari *et al* (30) reported the microRNA profiling of colon cancer cells, but the colon cancer cells were from colon cancer tissues. They did not report the microRNA profiling of SP cells from colon cancer. In the present study, we were able to isolate SP cells from all three colon cancer cell lines (HCT15, HT29, LoVo). The ratio of SP cells in the HCT-15, HT-29 and LoVo colon cancer cell lines was 16.75, 13.02 and 9.52%, respectively. Inoda *et al* (31) reported that the ratio of SP cells in the HCT-15, HT-29 and LoVo colon cancer cell lines was 11.1, 10.4 and 9.1%, respectively. Our data were similar to Inoda's results. This indicates that each type of colon cancer cell line contains a certain ratio of SP cells.

SP and non-SP cells sorted from the HCT-15, HT-29 and LoVo colon cancer cell lines were treated with different concentrations of 5-fluorouracil, oxaliplatin and adriamycin, respectively, for 3 days. Our data showed that cell viability of the SP cells derived from whichever HCT-15, HT-29 or LoVo colon cancer cell line used, was significantly higher than that of the non-SP cells at the same concentration of 5-fluorouracil. This indicated that the SP cells of the colon cancer cell lines were more resistant to 5-fluorouracil. Similar results were found in the presence of oxaliplatin and adriamycin. Therefore the SP cells of the colon cancer cell lines were more resistant to antitumor drugs. Inoda *et al* reported that SP cells from colon cancer cell lines SW480, HT-29 and HCT-15 showed resistance to chemotherapeutic agents such as irinotecan or

etoposide (31). All of these findings indicate that SP cells of colon cancer cells are more resistant to chemotherapeutic drugs than non-SP cells. Therefore, SP cells play an important role in multidrug resistance of colon cancer.

Since SP cells play an important role in multidrug resistance of colon cancer, we aimed to investigate the specific biomarkers of SP cells. In a previous study, Behbod *et al* investigated the gene markers of SP cells in breast cancer (19). Microarray gene profiling suggests that SP cells in breast cancer are a lineage-deficient mammary gland subpopulation expressing key genes involved in cell cycle regulation, development and angiogenesis. In the present study, we performed the microRNA profiling of SP cells in colon cancer cell lines to investigate the microRNA biomarkers of SP cells in colon cancer. miRCURY™ LNA Array (v.18.0), the newest version of microRNA array which can simultaneously detect hundreds of microRNAs, was used to perform the microRNA profiling of SP cells in colon cancer cell lines. In the HCT-15, HT-29 and LoVo colon cancer cell lines, three microRNAs including miR-5000-3p, miR-5009-3P and miR-552 were upregulated in SP cells of all of these three colon cancer cell lines. However, no microRNA was downregulated in SP cells of all three colon cancer cell lines. Furthermore, the findings were also confirmed by RT-PCR. In previous studies, Schetter *et al* (29) and Callari *et al* (30) reported the microRNA profiling of colon cancer cells. However, the colon cancer cells were from colon cancer tissues. Link *et al* (32) reported fecal microRNA profiling of colon cancer whereas the stool samples were from patients with colon cancer and healthy volunteers. Hofsli *et al* (33) investigated the serum microRNA profile of colon cancer while the serum samples were from patients with colon cancer and healthy controls. Although Zhang *et al* (34) and Fang *et al* (35) investigated the microRNA expression profile of colon cancer stem-like cells, they collected colon cancer stem-like cells based on CD133 or CD133/CD44 not SP cells. In addition to this, they compared the microRNA expression profile of colon cancer stem cells with non-stem cells only using one type of colon cancer cell line. No study on the microRNA profiling of SP cells of colon cancer was reported in all of the above studies. Therefore, the present study was the first report on microRNA profiling of SP cells in colon cancer.

In the present study, three microRNAs were found to be upregulated in SP cells of all three colon cancer cell lines including HCT-15, HT-29 and LoVo. These three microRNAs including miR-5000-3p, miR-5009-3P and miR-552 may be potential microRNA biomarker candidates of SP cells in colon cancer. As we know, SP cells play an important role in multidrug resistance of colon cancer. Thus, these three microRNAs may also be potential targets for the treatment of colon cancer. In future research, antisense RNA targeted to one of these three microRNAs will be used to inhibit the specific microRNA expression to investigate whether antisense RNA targeting to miR-5000-3p, miR-5009-3P or miR-552 reverses the multidrug resistance of colon cancer.

Acknowledgements

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