miR-214 reduces cisplatin resistance by targeting netrin-1 in bladder cancer cells

JIAO LIU, JIANBIN BI, ZELIANG LI, ZHENHUA LI, XIANKUI LIU and CHUIZE KONG

Department of Urology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

Received January 22, 2017; Accepted January 8, 2018

DOI: 10.3892/ijmm.2018.3374

Abstract. miR-214 has been reported to be downregulated in several cancer types, such as bladder cancer. However, its involvement in apoptosis and chemoresistance has not been investigated. The present study aimed to clarify the biological function of miR-214 and potential mechanisms in chemoresistance of bladder cancer cells. Reverse transcription-quantitative polymerase chain reaction demonstrated that miR-214 was downregulated in bladder cancer tissues compared with the level in normal tissues. miR-214 was downregulated in bladder cancer cell lines compared with the level in the normal cell line SV-HUC-1. miR-214 mimics were transfected into T24 and J82 cell lines to restore its expression. The results indicated that miR-214 mimic inhibited proliferation and invasion in these cell lines. In addition, miR-214 mimic reduced cisplatin resistance in T24 and J82 cells, indicated by the inhibition of cell viability and upregulation of cell apoptosis. Western blotting demonstrated that miR-214 mimic was able to upregulate cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP), while downregulate caspase-3 and PARP expression, and AKT phosphorylation. Using prediction software, it was revealed that the netrin-1 oncoprotein is on the target list of miR-214. miR-214 also downregulated netrin-1 protein and mRNA expression levels in the T24 and J82 cell lines. Luciferase reporter assays demonstrated that netrin-1 acted as a direct target of miR-214. A negative correlation between netrin-1 and miR-214 expression in bladder cancer tissues was also observed. In addition, cisplatin treatment could induce netrin-1 protein expression in bladder cancer cells and miR-214 mimic partly blocked this phenomenon. Netrin-1 plasmid transfection inhibited cisplatin-induced apoptosis, upregulated AKT phosphorylation, and downregulated caspase-3 and PARP cleavage. Netrin-1 was restored in cells transfected with miR-214 mimic using plasmid transfection. Netrin-1 transfection restored AKT phosphorylation and blocked caspase/PARP cleavage in the T24 and J82 cell lines. In conclusion, the present study demonstrated that miR-214 is downregulated in bladder cancer tissues and cell lines. miR-214 reduces chemoresistance by targeting netrin-1 in bladder cancer cell lines.

Correspondence to: Dr Chuize Kong, Department of Urology, The First Affiliated Hospital of China Medical University, 155 Nanjing Street, Shenyang, Liaoning 110001, P.R. China
E-mail: mskongchuize@sina.com

Key words: bladder cancer, miR-214, Netrin-1, proliferation, chemoresistance

Introduction

Bladder cancer is one of the most common malignant tumors of the urological system, and is also one of the main causes of cancer-related mortality (1). The prognosis of bladder cancer is dependent on its potential to invade and the development of chemoresistance (2-4). There are several risk factors for bladder cancer, including smoking, environmental and occupational exposure and gender (5). Although combined therapies, including surgery and chemotherapy, have been developed over the past decades, the prognosis for bladder cancer in the advanced stage remains poor (6). The identification of novel molecular targets and therapeutic strategies are required in order to achieve long-term survival of patients. Resistance to chemotherapy drives the search for identification of more effective targets to overcome chemoresistance.

MicroRNA (miRNA) are small non-coding RNA that downregulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNA (7,8). miRNA have been reported to be dysregulated in various types of human cancer, including lung, colorectal and bladder cancer, and they serve important roles in carcinogenesis, invasion, metastasis and development of chemoresistance (9-11).

miR-214 is regarded as a tumor suppressor that is downregulated in various cancer types, including breast, lung and colorectal carcinoma (12-14). The expression status of miR-214 was also reported in bladder cancer (15). miR-214 was downregulated in bladder cancer tissues and significantly associated with tumor stage, lymph node status and tumor grade (15). miR-214 may serve as an independent factor of recurrence-free survival and overall survival for patients with muscle-invasive bladder cancer. Urinary levels of cell-free miR-214 could be an independent prognostic parameter for non-muscle-invasive bladder cancer recurrence (16). miR-214 exerts tumor-suppressive effects in bladder cancer by downregulating oncogenic P53 and DNA damage regulated 1 (PDRG1) expression (15). Currently, the role of miR-214 in cancer cell apoptosis and drug resistance remains unclear. To

clarify this issue, the present study transfected miR-214 mimics into human bladder cancer cell lines and examined its role in cisplatin resistance. The underlying molecular mechanism was also explored.

Materials and methods

Clinical specimens. Fresh bladder cancer samples and corresponding normal adjacent tissues were obtained from 30 patients (22 males and 8 females; age, 56.1±11.6 years) at The First Affiliated Hospital of China Medical University (Shenyang, China) between September 2013 and August 2015. The present study was conducted with the approval of the Ethics Committee of China Medical University. Written informed consent was obtained from all patients and bladder cancer was confirmed by pathological diagnosis.

Cell culture and transfection. SV-HUC-1, RT-4, J82 and T24 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. miR-214 mimic (5'-ACA GCAGGCACAGACAGGCAGU-3') and corresponding control were obtained from Guangzhou RiboBio Co., Ltd., (Guangzhou, China). Cells were transfected with 50 nM concentration. Netrin-1 plasmid (pCMV6-NTN1) was obtained from OriGene Technologies, Inc. (Rockville, MD, USA). Plasmid was transfected at 1 μg/ml concentration. miR-214 mimic was transfected using DharmaFECT 1 Reagent (GE Healthcare, Chicago, IL, USA). Lipofectamine 3000 reagent was used for plasmid transfection (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested for subsequent experiments 4 h after transfection. For cell viability and apoptosis assays, cells were treated with cisplatin (10 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24, 48 and 72 h at 37˚C in 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from tissues and cells using RNAiso plus (Takara Biotechnology Co., Ltd., Dalian, China). For quantification of miR-214, primers (Guangzhou RiboBio Co., Ltd.) for miR-214 (Bulge-LoopTM miRNA qRT-PCR Primer Set for hsa-mir-214) and U6 (U6 small nuclear RNA qRT-PCR Primer) were used. Reverse transcription (37˚C for 15 min and 85˚C for 5 sec) was performed using a PrimeScript RT reagent kit from Takara Biotechnology Co., Ltd. qPCR was performed using ABI SYBR-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Analytik Jena QTOWER PCR system (Analytik Jena AG, Jena, Germany), according to the manufacturer's protocol. The thermocyclying conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The relative levels of gene expression were represented as ΔCt=Ct miR-214-Ct U6. The change of gene expression was calculated by the $2^{-\Delta\Delta Cq}$ method (15).

For quantification of netrin-1 mRNA, total RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.). qPCR was performed using an ABI SYBR Green MasterMix on an Analytik Jena QTOWER PCR system, according to the manufacturer's protocol. The thermocyclying conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The relative levels of gene expression were represented as ΔCt=Ct gene-Ct reference, and the fold change of gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (15). The primer sequences used were as follows: Netrin-1, forward 5'-GTCAATGCGGCCTTCGG-3' and reverse 5'-CTGCTC GTTCTGCTTGGTGAT-3'; and β-actin, forward 5'-ATAGCA CAGCCTGGATAGCAACGTAC-3' and reverse 5'-CACCTT CTACAATGAGCTGCGTGTG-3'.

Western blot analysis. Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) and quantified using the Bradford method. Protein samples (~40 μg) were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. Blocking was performed using 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at room temperature for 2 h. The membrane was incubated at 4˚C overnight with primary antibodies, including netrin-1 (1:500; ab126729; Abcam, Cambridge, MA, USA), caspase-3 (9665), cleaved caspase-3 (9664), poly (ADP-ribose) polymerase (PARP) (9532), cleaved PARP (5625), phosphorylated (p)-AKT (4060), AKT (4691) B-cell lymphoma (Bcl)-2 (15071) (all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (sc-32233) (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following incubation with horseradish peroxidase-conjugated secondary antibody (7071/7072; 1:2,000; Cell Signaling Technology, Inc.) at 37˚C for 2 h, visualization was performed using an enhanced chemiluminescent kit (Pierce; Thermo Fisher Scientific, Inc.) and a DNR Bio-Imaging System (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel).

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assay was conducted to detect cell viability using a CCK-8 kit from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA), according to the manufacturer's protocol. Briefly, cultured cells (2,000 cells/well) were incubated with 10 μl/well CCK-8 solution for 4 h at 37˚C in an incubator. Subsequently, the optical density of each well was measured at a wavelength of 490 nm.

Colony formation assay. Cells were seeded into three 6-cm cell culture dishes with DMEM supplemented with 10% FBS (1,000 cells/dish) and incubated at 37˚C in 5% CO_2 for at least 2 weeks. Subsequently, the plates were washed with PBS and fixed using cold 100% methanol at 4˚C for 10 min. Following this, the plates were stained with Giemsa at room temperature for 30 min. Colony number was counted manually using an Olympus CKX41 light microscope (Olympus Corporation, Tokyo, Japan) with a magnification of x100.

Matrigel invasion assay. A Matrigel invasion assay was conducted using a 24-well Transwell chamber from Costar (Corning Incorporated, Corning, NY, USA) coated with 20 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), with a

dilution rate of 1:8. A total of 100,000 cells were transferred into the upper Transwell chamber in DMEM without serum and incubated for 18 h to allow the cells to invade. Remaining cells were wiped out using a cotton tip and invading cells were fixed with 4% paraformaldehyde at room temperature for 10 min and stained with hematoxylin at room temperature for 10 min. Colony number was counted using an Olympus BX53 light microscope (Olympus Corporation) under a magnification of x200.

Flow cytometry for apoptosis. The apoptosis rate was detected using an Annexin V/propidium iodide (PI) staining kit (BD Biosciences), according to the manufacturer's protocol. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and data was analyzed using FlowJo v. 10 software (FlowJo LLC, Ashland, OR, USA).

Validation of target gene and miRNA interaction. A p-MIR-reporter vector (Thermo Fisher Scientific, Inc.) was used for 3'-UTR luciferase reporter assays to determine the interaction of miR-214 with netrin-1. The wild-type (WT) miR-214 target site in netrin-1 3'-UTR was CCUGCUG. The mutant (MUT) miR-214 target site was CCUUUUG. TargetScan (targetscan.org) was used to identify miR-214 targets.

Cells with 80% confluence were co-transfected with the firefly luciferase reporter plasmid (0.1 μg) along with the reference *Renilla* luciferase reporter plasmid (0.01 μg) using Lipofectamine 3000, according to the protocols provided by the manufacturers. After 48 h of transfection, the luciferase activity was measured using a Promega Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). The relative activity of the reporter gene was calculated by dividing the signals from firefly luciferase reporter by the signals obtained from *Renilla* luciferase reporter.

Statistical analysis. Data were presented as the mean ± standard deviation, and experiments were repeated in triplicates. SPSS v. 16 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. A Student's t-test was used to compare data between control and treatment groups. One-way analysis of variance with Bonferroni's post hoc analysis was used to compare the means of more than two groups. All P-values were based on a two-sided statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-214 expression is downregulated in bladder cancer. The expression of miR-214 was examined in 30 samples of fresh bladder carcinoma tissues with corresponding normal tissues by RT-qPCR. As demonstrated in Fig. 1, miR-214 expression levels were significantly downregulated in cancer tissues compared with normal tissues. miR-214 cancer/normal ratio <0.5 was regarded as significant downregulation. It was revealed that miR-214 expression was significantly downregulated compared with the level in normal tissue in 13 out of 30 samples (significant was defined as ratio of cancer tissue/normal tissue <1/2) (Fig. 1A). The mean value of miR-214 expression between cancer and normal tissues was examined and it was demonstrated that the mean miR-214 expression level in normal tissue was significantly higher than that in cancer tissue (P<0.05; Fig. 1B). In addition, the association between miR-214 expression and T stage was evaluated. As demonstrated in Table I, the rate of miR-214 downregulation was 58.3% in T3+T4 bladder cancer and 33.3% in T1+T2 cancer, suggesting that miR-214 may be downregulated in cancer with a higher T stage. However, the difference did not reach a statistical significance.

Table I. Distribution of miR-214 expression in bladder cancer according to T status.

		miR-214 expression level		
Tumor local invasion status	Number of patients	Low	High	P-value
T1+T2	18	6	12	0.264
T3+T4	12	7	5	

miR, microRNA.

miR-214 is downregulated in bladder cancer cell lines and inhibits cell proliferation and invasion. The expression level of miR-214 in normal epithelial cell line SV-HUC-1 and three bladder cancer cell lines (J82, J82 and T24) was investigated. Expression of miR-214 was significantly lower in all bladder cancer cell lines compared with that in SV-HUC-1 cells (P<0.001; Fig. 2A). The T24 and J82 cell lines, with low endogenous expression, were selected for transfection with miR-214 mimic. The transfection efficiency was confirmed by RT-qPCR. Transfection with miR-214 mimic significantly increased the expression level of miR-214 in J82 and T24 cells compared with the level in the mimic control group (P<0.05; Fig. 2A). Using a colony formation assay, it was demonstrated that miR-214 mimic significantly reduced the colony formation ability of the J82 and T24 cell lines compared with that observed in the mimic control group (P<0.05; Fig. 2B). Matrigel invasion assays indicated that miR-214 mimic significantly inhibited the invading ability of the J82 and T24 cell lines compared with that observed in the mimic control group (P<0.05; Fig. 2C).

miR-214 reduces cisplatin resistance and inhibits AKT phosphorylation. The role of miR-214 on chemoresistance of bladder cancer cells was explored. A CCK-8 assay was conducted in T24 and J82 cells treated with cisplatin (10 μg/ml). As demonstrated in Fig. 3A, miR-214 mimic significantly reduced cell viability in both cell lines at 24, 48 and 72 h following treatment with cisplatin compared with that observed in the mimic control group (P<0.05). These results suggest that miR-214 reduced chemoresistance of bladder cancer cells. Annexin V/PI staining was conducted to measure the rate of

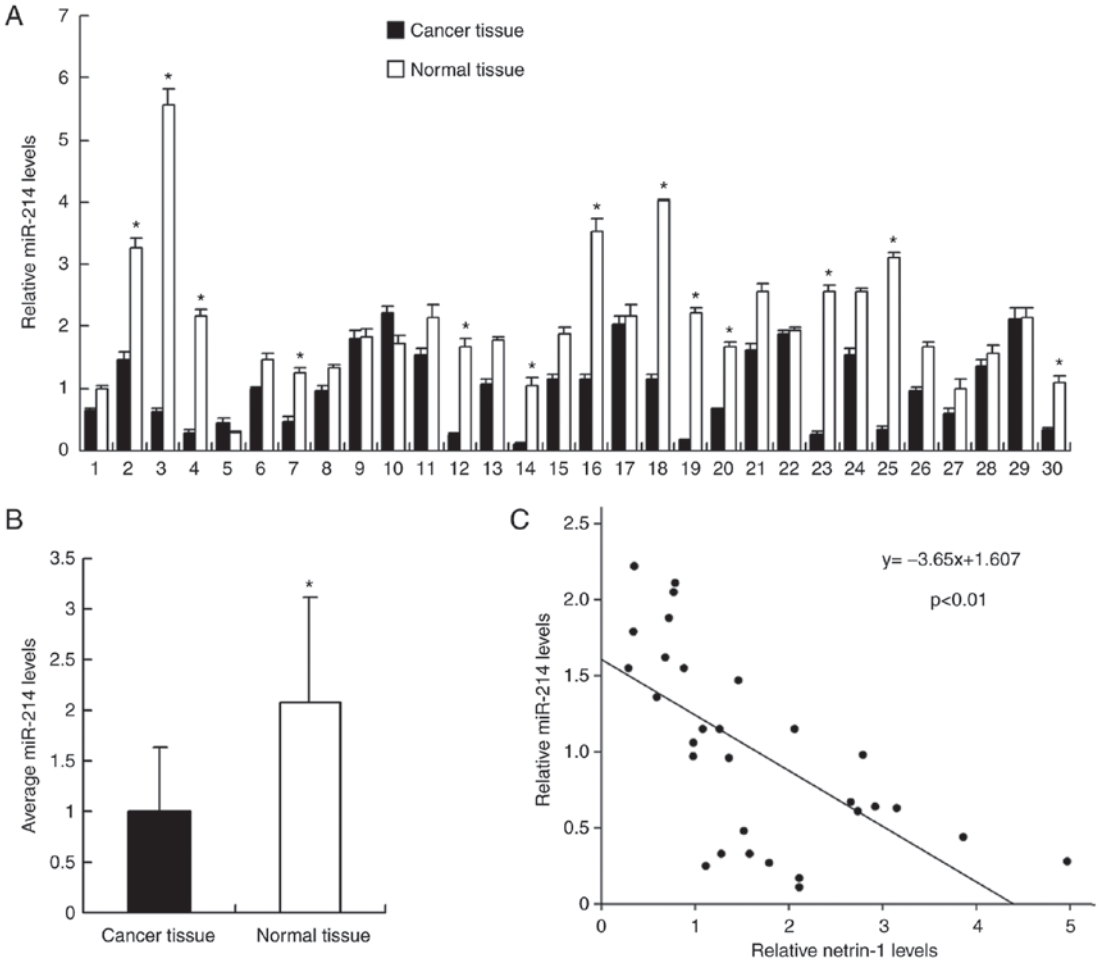

Figure 1. Expression pattern of miR-214 in bladder cancer tissues. (A) Relative expression level of miR-214 in 30 fresh bladder cancer tissues and paired normal tissues. (B) The mean miR-214 expression level in bladder cancer tissues was lower than that in corresponding normal tissues. (C) A negative correlation was identified between miR-214 and netrin-1 mRNA expression levels in bladder cancer tissues using linear regression analysis. *P<0.05 vs. cancer tissue. miR, microRNA.

apoptosis. As indicated in Fig. 3B, transfection with miR-214 mimic significantly upregulated the apoptosis rate after 24 h of cisplatin treatment in the J82 and T24 cell lines. Western blotting was performed to examine the change of related proteins. As indicated in Fig. 3C, transfection with miR-214 mimic downregulated total caspase-3 and total PARP expression, and upregulated cleaved caspase-3 and cleaved PARP. Several signaling pathways involved in cancer cell survival were also analyzed, and it was revealed that miR-214 inhibited AKT phosphorylation.

miR-214 targets and downregulates netrin-1. TargetScan software was used to predict potential targets of miR-214, and this revealed that netrin-1 is one of the target genes (17). To validate the relationship between netrin-1 and miR-214, the change in expression level of netrin-1 mRNA and protein was examined following transfection with miR-214 mimic. As demonstrated in Fig. 4A, transfection with miR-214 mimic markedly and significantly downregulated netrin-1 expression at the protein and mRNA levels, respectively, compared with the level in the mimic control group (P<0.05; Fig. 4A).

To validate if netrin-1 is the direct target of miR-214, a luciferase reporter assay was conducted. Reporters with wild-type (CCUGCUG) and mutant (CCUUUUG) 3'-UTR binding sites of netrin-1 were introduced into J82 cells together with miR-214 mimic. The luciferase reporter assay demonstrated that miR-214 mimic significantly suppressed the luciferase intensity of the wild-type reporter compared with that in the control group (P<0.05); however, no significant change was observed in the luciferase intensity of the mutant reporter (Fig. 4B). The above data indicates that miR-214 binds to the 3'-UTR of netrin-1 to reduce its mRNA and protein expression. In addition, the correlation between miR-214 and netrin-1 mRNA expression in bladder cancer tissues was examined. Linear regression analysis demonstrated that the miR-214 expression level in cancer tissues was negatively correlated with netrin-1 mRNA expression (P<0.01; Fig. 1C).

Cisplatin treatment induces netrin-1 expression in bladder cancer cells. Chemotherapeutic agents, including cisplatin and doxorubicin, have been reported to induce netrin-1 expression, which appears to be a survival mechanism, as the depletion of netrin-1 results in cancer cell death (18). To confirm this, the alteration of the netrin-1 expression level in bladder cancer cells following cisplatin treatment was investigated. As demonstrated in Fig. 5, cisplatin treatment (2 and 5 μg/ml for

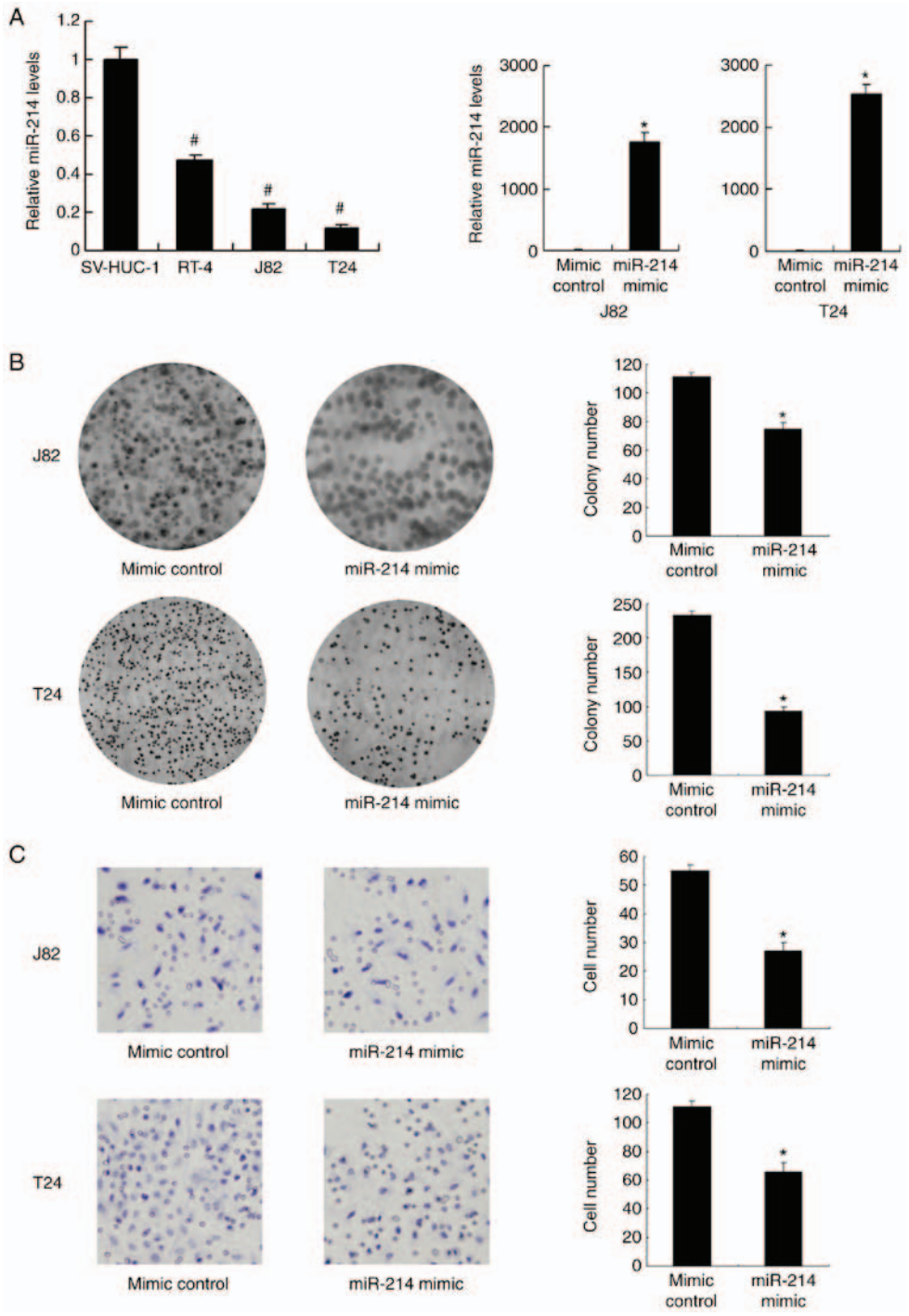

Figure 2. Effects of miR-214 on cell proliferation in bladder cancer cell lines. (A) Expression level of miR-214 in three bladder cancer cell lines and a normal cell line, SV-HUC-1. miR-214 mimic upregulated miR-214 expression levels in T24 and J82 cell lines. (B) miR-214 mimic decreased the colony number of T24 and J82 cell lines (magnification, x5). (C) miR-214 mimic decreased the invading ability of T24 and J82 cell lines (magnification, x400). #P<0.001 vs. SV-HUC-1 cells; *P<0.05 vs. mimic control. miR, microRNA.

2 days) induced an increase of protein expression in the J82 and T24 cell lines compared with the level in cells that did not receive cisplatin treatment. At the mRNA expression level, this increase was significant in cells treated with 5 μg/ml cisplatin (P<0.05). mRNA expression levels of netrin-1 increased significantly following treatment with 5 μg/ml cisplatin, which was confirmed by one-way analysis of variance with post hoc comparisons in both cell lines (J82: 5 μg/ml vs. control, P=0.004 and 5 vs. 2 μg/ml, P=0.006; T24: 5 μg/ml vs. control, P=0.001 and 5 vs. 2 μg/ml, P=0.002; Fig. 5).

miR-214 regulates cisplatin resistance through netrin-1. To validate the biological function of netrin-1, netrin-1 was overexpressed in T24 and J82 cell lines. As demonstrated in Fig. 6A, Annexin V/PI analysis indicated that netrin-1 overexpression significantly inhibited cisplatin-induced apoptosis compared with the level in the control group (P<0.05). Western blotting indicated that netrin-1 transfection upregulated AKT phosphorylation. These data suggest that netrin-1 is associated with chemoresistance in bladder cancer cells. To confirm the involvement of netrin-1 in the biological effects

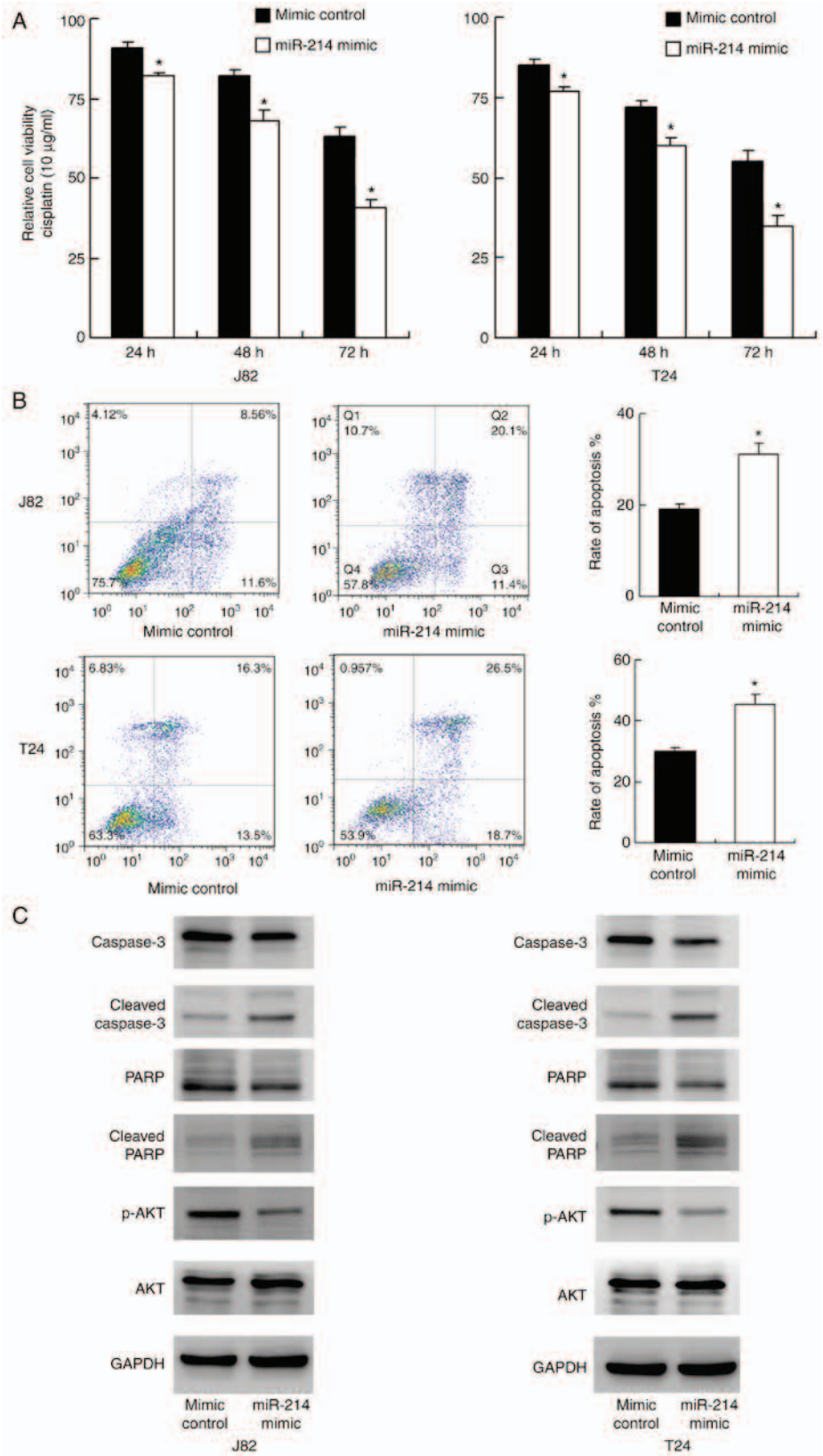

Figure 3. miR-214 decreases cisplatin resistance. (A) The viability of cancer cells treated with cisplatin was measured using a Cell Counting Kit-8 assay. miR-214 mimic transfection increased sensitivity to cisplatin in T24 and J82 cells during 72 h of treatment. (B) miR-214 mimic transfection upregulated the apoptosis rate in T24 and J82 cells after 48 h of treatment. (C) miR-214 mimic downregulated total caspase-3, total PARP and p-AKT expression, with upregulation of cleaved caspase-3 and cleaved PARP. *P<0.05 vs. mimic control. miR, microRNA; PARP, poly (ADP-ribose) polymerase; p, phosphorylated.

of miR-214, netrin-1 plasmid transfection was conducted in bladder cancer cells with miR-214 mimic. As indicated in Fig. 6B, plasmid transfection restored netrin-1 and p-AKT status in cells transfected with miR-214 mimic, with downregulation of caspase/PARP cleavage. In addition, the expression of the AKT target protein, Bcl-2, was studied. miR-214 mimic

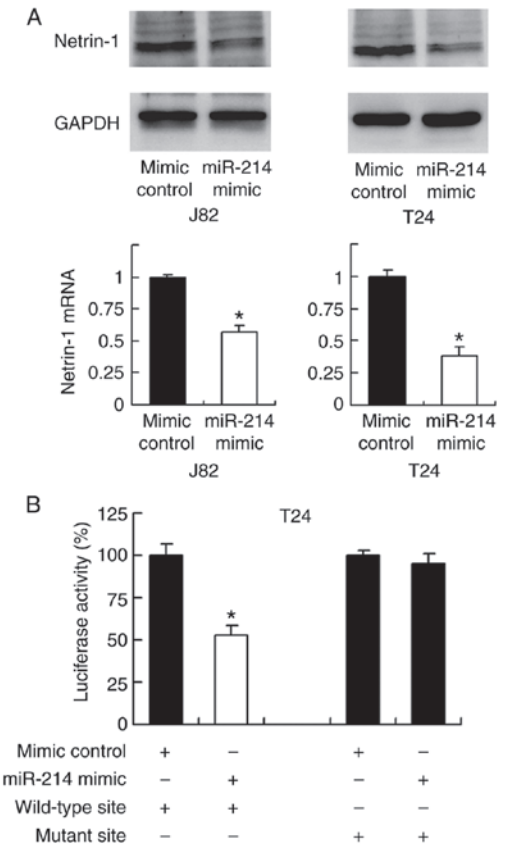

Figure 4. Netrin-1 is a direct target of miR-214 in bladder cancer cells. (A) Western blotting and reverse transcription-polymerase chain reaction analysis demonstrated that miR-214 mimic downregulated the protein and mRNA expression levels of netrin-1. (B) Fluorescence reporter plasmids with wild-type or mutant binding sites were introduced into T24 cells with miR-214 mimic. In cells transfected with wild-type reporter, the miR-214 mimic downregulated the luciferase activity. In cells transfected with the mutant site reporter, no significant change was observed. *P<0.05 vs. mimic control. miR, microRNA.

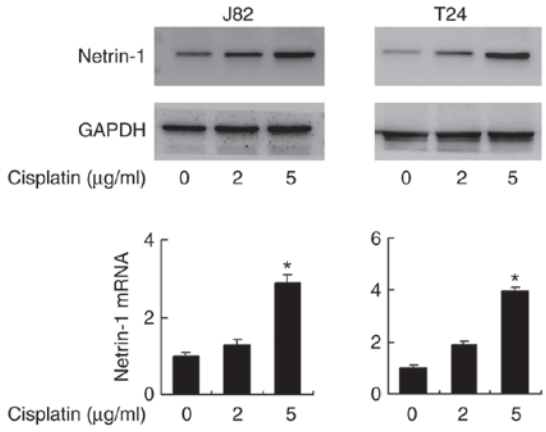

Figure 5. Cisplatin treatment induces netrin-1 expression in bladder cancer cells. In T24 and J82 cell lines, cisplatin treatment (2 and 5 μg/ml for 2 days) increased netrin-1 mRNA and protein expression levels in a concentration-dependent manner. *P<0.05 vs. 0 and 2 μg/ml cisplatin.

downregulated Bcl-2 expression and netrin-1 transfection restored Bcl-2 status. Together, these data demonstrate that the function of miR-214 in chemoresistance is, at least partly, through netrin-1 regulation.

Discussion

Accumulating evidence has demonstrated the role of miRNA during cancer development and progression. miR-214 is regarded as a tumor suppressor that is downregulated in various cancer types, including breast, lung and colorectal carcinoma (15,19-21). The expression status of miR-214 has been reported to be downregulated in bladder cancer tissues and significantly associated with tumor stage, lymph node status and cancer grade (15). Urinary levels of cell-free miR-214 could be an independent prognostic parameter for non-muscle-invasive bladder cancer recurrence (16). miR-214 exerts tumor-suppressive effects in bladder cancer by down-regulating oncogenic PDRG1 expression (15). In the present study, it was demonstrated that miR-214 expression levels are downregulated in bladder cancer tissues and cell lines. The suppressive effects of miR-214 on bladder cancer proliferation and invasion were also validated in the present study. However, its involvement in the development of chemoresistance of bladder cancer cells had yet to be explored.

Thus, the present study investigated the influence of miR-214 on resistance to chemotherapy by using cisplatin treatment in J82 and T24 cells. The results demonstrated that miR-214 mimic significantly reduced drug resistance by upregulating the apoptosis rate. A series of apoptosis-related proteins were examined. The present results revealed that total caspase-3 and total PARP expression levels were decreased following transfection with miR-214 mimic, while cleaved caspase-3 and cleaved PARP were markedly upregulated. These results indicate that miR-214 promotes apoptosis. In addition, the present study indicated that miR-214 was able to suppress AKT phosphorylation. AKT signaling is involved in the regulation of cell apoptosis by upregulating pro-survival Bcl-2 family proteins, including Bcl-2 and Bcl-extra large (22). Thus, the effects of miR-214 on chemoresistance may be dependent on its regulation of AKT/Bcl-2 signaling.

The present study further explored the mechanism by which miR-214 regulates apoptosis and chemoresistance. Potential target genes of miR-214 were predicted using TargetScan software, and netrin-1 was identified as one of the targets. Netrin-1 has been identified as an oncoprotein in several cancer types, such as bladder cancer (23). Previously we reported that netrin-1 overexpression was associated with poor prognosis in patients with bladder cancer (23). A previous report suggested that netrin-1 may enhance chemoresistance of cancer cells through regulation of its receptor activity (19). In the present study, the effects of netrin-1 on bladder cancer apoptosis and AKT signaling were investigated. The results demonstrated that netrin-1 plasmid transfection significantly downregulated apoptosis and notably upregulated AKT phosphorylation in bladder cancer cells. In addition, miR-214 mimic significantly and markedly decreased netrin-1 mRNA and protein expression levels, respectively. Notably, a negative relationship was identified in bladder cancer tissues between miR-214 and netrin-1 mRNA expression levels in the present study. Furthermore, luciferase reporter assays revealed that miR-214 could directly bind to the 3'-UTR of netrin-1, demonstrating that netrin-1 is a direct target of miR-214. These results together suggest that miR-214 downregulates netrin-1, which in turn inhibits p-AKT and

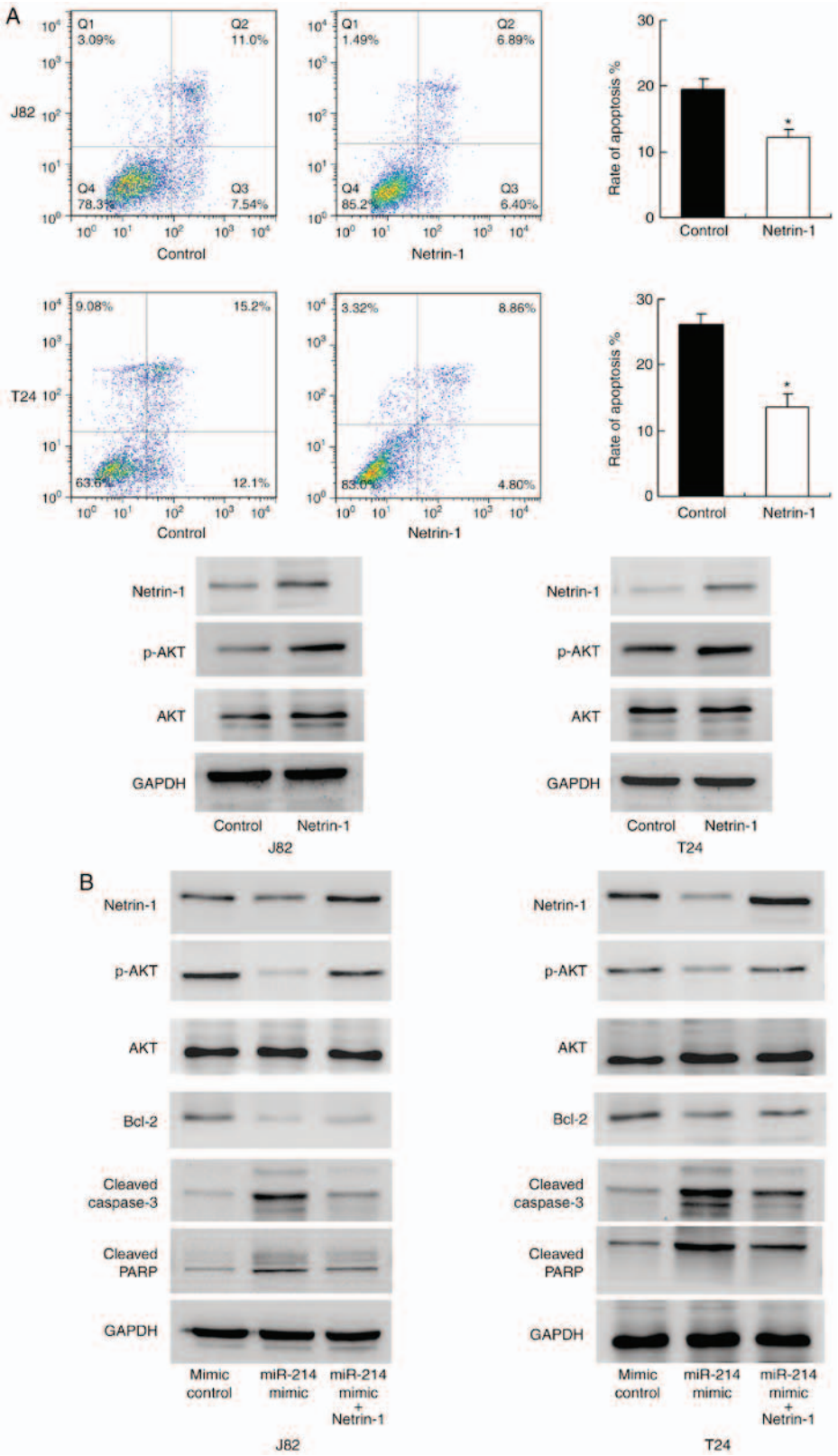

Figure 6. miR-214 regulates cisplatin resistance through netrin-1. (A) Annexin V/propidium iodide analysis demonstrated that netrin-1 overexpression inhibited cisplatin-induced apoptosis. Western blotting indicated that netrin-1 transfection upregulated AKT phosphorylation. (B) Netrin-1 plasmid transfection restored netrin-1 and p-AKT protein and downregulated cleaved caspase-3 and cleaved-PARP expression in T24 and J82 cells transfected with miR-214 mimic. Transfection with miR-214 mimic also downregulated Bcl-2 expression in both cell lines. *P<0.05 vs. control. miR, microRNA; PARP, poly (ADP-ribose) polymerase; p, phosphorylated; Bcl-2, B-cell lymphoma 2.

reduces chemoresistance. To further confirm this, netrin-1 plasmid was used to restore its function in the present study. Netrin-1 plasmid transfection restored cisplatin resistance downregulated by miR-214. Taken together, these data suggest that miR-214 inhibits chemoresistance in bladder cancer by targeting netrin-1.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate the relationship between miR-214 and chemoresistance of bladder cancer cells. miR-214 may reduce cisplatin resistance and AKT signaling by targeting netrin-1. The present findings may contribute to an improved understanding of the mechanism involved in bladder cancer chemoresistance.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81402088).

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.
2. Reddy OL, Cates JM, Gellert LL, Crist HS, Yang Z, Yamashita H, Taylor JA III, Smith JA Jr, Chang SS, Cookson MS, *et al*: Loss of FOXA1 drives sexually dimorphic changes in urothelial differentiation and is an independent predictor of poor prognosis in bladder cancer. Am J Pathol 185: 1385-1395, 2015.
3. Szarvas T, László V, Vom Dorp F, Reis H, Szendröi A, Romics I, Tilki D, Rübben H and Ergün S: Serum endostatin levels correlate with enhanced extracellular matrix degradation and poor patients' prognosis in bladder cancer. Int J Cancer 130: 2922-2929, 2012.
4. Yang GL, Zhang LH, Bo JJ, Huo XJ, Chen HG, Cao M, Liu DM and Huang YR: Increased expression of HMGB1 is associated with poor prognosis in human bladder cancer. J Surg Oncol 106: 57-61, 2012.
5. Dobruch J, Daneshmand S, Fisch M, Lotan Y, Noon AP, Resnick MJ, Shariat SF, Zlotta AR and Boorjian SA: Gender and bladder cancer: A collaborative review of etiology, biology, and outcomes. Eur Urol 69: 300-310, 2016.
6. Mitra AP: Molecular substratification of bladder cancer: Moving towards individualized patient management. Ther Adv Urol 8: 215-233, 2016
7. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, *et al*: MicroRNA expression profiles classify human cancers. Nature 435: 834-838, 2005.
8. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, *et al*: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64: 3753-3756, 2004.
9. Zhang DQ, Zhou CK, Jiang XW, Chen J and Shi BK: Increased expression of miR-222 is associated with poor prognosis in bladder cancer. World J Surg Oncol 12: 241, 2014.
10. Kohler CU, Bryk O, Meier S, Lang K, Rozynek P, Brüning T and Käfferlein HU: Analyses in human urothelial cells identify methylation of miR-152, miR-200b and miR-10a genes as candidate bladder cancer biomarkers. Biochem Biophys Res Commun 438: 48-53, 2013.
11. Puerta-Gil P, Garcia-Baquero R, Jia AY, Ocaña S, Alvarez-Múgica M, Alvarez-Ossorio JL, Cordon-Cardo C, Cava F and Sánchez-Carbayo M: miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. Am J Pathol 180: 1808-1815, 2012.
12. Zhang J, Su B, Gong C, Xi Q and Chao T: miR-214 promotes apoptosis and sensitizes breast cancer cells to doxorubicin by targeting the RFWD2-p53 cascade. Biochem Biophys Res Commun 478: 337-342, 2016.
13. Yu X, Luo A, Liu Y, Wang S, Li Y, Shi W, Liu Z and Qu X: MiR-214 increases the sensitivity of breast cancer cells to tamoxifen and fulvestrant through inhibition of autophagy. Mol Cancer 14: 208, 2015.
14. Li QQ, Xie YK, Wu Y, Li LL, Liu Y, Miao XB, Liu QZ, Yao KT and Xiao GH: Sulforaphane inhibits cancer stem-like cell properties and cisplatin resistance through miR-214-mediated downregulation of c-MYC in non-small cell lung cancer. Oncotarget 8: 12067-12080, 2017.
15. Wang J, Zhang X, Wang L, Yang Y, Dong Z, Wang H, Du L and Wang C: MicroRNA-214 suppresses oncogenesis and exerts impact on prognosis by targeting PDRG1 in bladder cancer. PLoS One 10: e0118086, 2015.
16. Wang J, Zhang X, Wang L, Dong Z, Du L, Yang Y, Guo Y and Wang C: Downregulation of urinary cell-free microRNA-214 as a diagnostic and prognostic biomarker in bladder cancer. J Surg Oncol 111: 992-999, 2015.
17. Pan X, Peng G, Liu S, Sun Z, Zou Z and Wu G: MicroRNA-4649-3p inhibits cell proliferation by targeting protein tyrosine phosphatase SHP-1 in nasopharyngeal carcinoma cells. Int J Mol Med 36: 559-564, 2015.
18. Paradisi A, Creveaux M, Gibert B, Devailly G, Redoulez E, Neves D, Cleyssac E, Treilleux I, Klein C, Niederfellner G, *et al*: Combining chemotherapeutic agents and netrin-1 interference potentiates cancer cell death. EMBO Mol Med 5: 1821-1834, 2013.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
20. Long LM, He BF, Huang GQ, Guo YH, Liu YS and Huo JR: microRNA-214 functions as a tumor suppressor in human colon cancer via the suppression of ADP-ribosylation factor-like protein 2. Oncol Lett 9: 645-650, 2015.
21. Chen DL, Wang ZQ, Zeng ZL, Wu WJ, Zhang DS, Luo HY, Wang F, Qiu MZ, Wang DS, Ren C, *et al*: Identification of microRNA-214 as a negative regulator of colorectal cancer liver metastasis by way of regulation of fibroblast growth factor receptor 1 expression. Hepatology 60: 598-609, 2014.
22. Wang YB, Qin J, Zheng XY, Bai Y, Yang K and Xie LP: Diallyl trisulfide induces Bcl-2 and caspase-3-dependent apoptosis via downregulation of Akt phosphorylation in human T24 bladder cancer cells. Phytomedicine 17: 363-368, 2010.
23. Liu J, Kong CZ, Gong DX, Zhang Z and Zhu YY: PKC α regulates netrin-1/UNC5B-mediated survival pathway in bladder cancer. BMC Cancer 14: 93, 2014.