The biological effect and mechanism of the Wnt/β-catenin signaling pathway on malignant melanoma A375 cells

YUXIA LIN[1], FANGFEI WANG[1], QINGFEI XING[1], FENG GUO[1], MENGZHEN WANG[2] and YUNJIE LI[3]

Departments of [1]Urology, [2]Finance and [3]Equipment, Jinan Central Hospital, Jinan, Shandong 250013, P.R. China

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Abstract. The present study aimed to determine the influence of the Wnt/β-catenin signaling pathway on the proliferation, invasion, migration and apoptosis of malignant melanoma (MM) A375 cells. β-catenin interfering lentivirus liquid (β-catenin-RNAi-LV) and empty vector lentivirus liquid (β-catenin-negative-LV) were used to infect A375 cells. Infected cells were obtained and marked as A375-RNA interference (A375-RNAi) or A375-negative, respectively. Western blotting was used to measure the expression of β-catenin in infected cells and uninfected cells were utilized as a control. An MTT assay was adopted to measure cell proliferation and the clone formation of cells was assessed. In addition, the Transwell method was used to detect cell invasion and migration *in vitro* and flow cytometry was utilized to determine cell apoptosis. Western blot analysis demonstrated that β-catenin was highly expressed in uninfected A375 cells but exhibited reduced expression in A375-RNAi cells. These results indicate that β-catenin expression is effectively silenced by β-catenin-RNAi-LV. The proliferative and clone forming abilities of A375-RNAi cells were impaired compared with A375-negative and A375 cells. Additionally, the apoptosis rate was increased and the invasion and migration of A375-RNAi cells was decreased. However, no significant differences were identified in the proliferation, clone formation, apoptosis rate, invasion and migration of A375-negative cells compared with A375 cells. Therefore, the current study demonstrated that the inhibition of β-catenin expression or activity inhibits cell proliferation and invasion and migration, further downregulating the expression of anti-apoptotic genes and accelerating cellular apoptosis.

Correspondence to: Dr Yunjie Li, Department of Equipment, Jinan Central Hospital, 105 Jiefang Road, Jinan, Shandong 250013, P.R. China
E-mail: dr_liyunjie@163.com

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Introduction

Malignant melanoma (MM) is a common malignant tumor of the skin that is increasing in incidence (1). MM, developed from moles or pigment spots, is fast growing and presents with a poor prognosis and a high mortality (2). Additionally, MM is more prevalent in adults (age, 18-60 years) and has an average survival period of ~30.3 months (2) due to it being prone to metastasis and recurrence (3,4). At present, the primary method of MM treatment is surgery as the tumor grows rapidly, meaning that it is often found in the growth (5). In cases where no distant metastasis is identified prior to surgery, MM may be fully resected with tumor margins removed; however, recurrence and metastasis may still result following surgery (6,7). Furthermore, postoperative adjuvant antitumor therapy has little effect on the prognosis of the disease (8). In addition to its strong propensity towards invasion and metastasis, MM is not sensitive to traditional anti-tumor therapy, including surgical excision (9). It has been demonstrated that signal transduction networks, particularly the Wnt/β-catenin pathway, serve crucial roles in the pathogenesis of MM (9). Previous studies have demonstrated that Wnt/n-catenin signaling serves a vital role in embryonic development, cell differentiation and proliferation and in the self-renewing capacity of stem and progenitor cells (10-12). In addition, it has been indicated that the Wnt/β-catenin pathway is involved in the regulation of neural crest melanophore formation and melanoblast development (13,14). The present study therefore examined the influence of β-catenin downregulation on the biological behavior of MM cells *in vitro*.

Materials and methods

Cell culture and transfection. The human MM A375 cell line was purchased from the Biovector Science Lab, Inc. (Beijing, China). There were 3 groups included in the present study: The experimental group consisted of interfering lentivirus infected A375 cells, marked as A375-RNAi; the negative control group consisted of A375 cells infected using a lentivirus empty vector, marked as A375-negative and the blank control group consisted of uninfected A375 cells, marked as A375. MM A375 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Beijing Transgen Biotech Co., Ltd., Beijing, China) containing 1% penicillin, streptomycin and

10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated at 37˚C and 5% CO_2. The medium was replenished every 2-3 days depending on cell growth, which was monitored using an inverted microscope. When 80-90% confluence was reached, cells were passaged. To achieve this, the medium was carefully discarded and the cells were washed 2-3 times with PBS prior to the addition of 1 ml 0.25% pancreatin (Shanghai Suer Biotechnology Co., Ltd., Shanghai, China). Cells were observed using an inverted microscope (magnification, x20) and when the cells had become rounded in shape but remained attached to the cell wall, 2 ml complete medium (Dulbecco's modified Eagles' medium; DMEM; Beijing Transgen Biotech Co., Ltd.) was added to terminate digestion. A 3 ml sterile straw was then used to lightly agitate the bottom of the Culture bottle of the culture flask to detach cells from their cell walls. A 3 ml sterile straw was utilized to gently blow the cell culture at the bottom wall of the flask, to ensure for complete wall separation.

The cell suspension was then centrifuged for 3 min at 252 x g 37˚C and the supernatant was then removed. A total of 3 ml complete medium was used to re-suspend the cells and an appropriate quantity of medium was subsequently added to the culture flask at a ratio of 1:3. (Cells were then marked, cultured and incubated. Following incubation, cells were then counted using a hemocytometer (Shanghai Biochemical Reagent Refinement Instrument Co., Ltd., Shanghai, China).) and sterile glass coverslips, on which the cells were mounted on. A total of four fields were counted. The following formula was used to calculate the total number of cells: Total cellular score of the counting block grids/4x10^4 (cells/ml). Lentivirus small interfering RNA (siRNA) packaging was completed by Cyagen Biosciences Inc. (Santa Clara, CA, USA). The interference sequences of β-catenin interfering lentivirus liquid (β-catenin-RNAi-LV) and β-catenin empty vector lentivirus liquid (β-catenin-negative-LV; negative control) utilized in the present study were 5'-GGATGTGGATACCTCCCAAGT-3' and 5'-TTCTCCGAACGTGTCACGT-3', respectively, the latter being used as a control. The multiplicity of infection value of infected A375 cells was ~100. siRNA was transfected as a volume of 100 μl and the time interval between transfection and subsequent experimentation was 24 h. Cells were then planted in the 24 hole culture medium; Shanghai Bai Li Biological, Shanghai, China) one day in advance, at a confluence of 30% with 0.45 ml of 24 hole culture medium. Using the SiRNA living transfection kit (Guangzhou Bobai Trading Co., Ltd., Guangzhou, China) (http://www.chem17.com/st301933/Product_21772496.html) efficiency was calculated using the formula: (Number of green fluorescent cells/total number of cells) x100. A375 cells in the logarithmic growth phase were harvested and counted, and the cell suspension was diluted to 1x10^4/ml. The A375 cells that demonstrated good growth (determined by the absence of suspension or microsuspension in the culture medium and even growth of cells) were routinely processed and counted, and the cell suspension was diluted to 1x10^4/ml. Following dilution, cells were seeded in three wells of a 6-well plate plate. A375 cells were incubated in one well, A375 cells + β-catenin-negative-LV were incubated in a second well and A375 cells + β-catenin-RNAi-LV were incubated in a third well. Each well contained 2x10^4 cells in 2 ml complete culture medium. Then, the cells were placed in an orifice plate (consisting of 3 holes with A375 seeded into A, A375 cell + β-catenin-negative-LV into B and A375 cell + β-catenin-RNAi-LV into hole C; invitrogen; Thermo Fisher Scientific, Inc.). When cell confluence reached 30-40%, cells were infected and observed using a fluorescent microscope (magnification, x200). (Shenzhen Topco Industrial Co., Ltd., Senzhen, China). Each of the three cell groups were observed 4 days following treatment using a fluorescent microscope (Shenzhen Topco Industrial Co., Ltd., Shenzhen, China) At 4 days following transfection, the level of fluorescence was observed under an inverted fluorescence microscope. When the transfection efficiency was >70%, cells in each of the three groups were passaged and cultured for 24 h at 5% CO_2 and 37˚C for use in subsequent experiments.

Western blotting. Cells in each group included in the current study were first digested and centrifuged. The supernatant was then removed and cells were re-suspended. Cells were inoculated in 6-cm cell culture dishes. During total cell protein extraction, cells were centrifuged at a speed of 36,800 x g for 10 min at 4˚C to analyze the expression of β-catenin and β-actin. Following gel electrophoresis, the gel was cut between 34 and 130 kDa to determine the interlude of the marker. The separated proteins were subsequently transferred onto a nitrocellulose membrane. The constant current was adjusted to 200 mA and the membrane was rotated for 90 min. The membranes were subsequently incubated with 1:1,000 primary antibodies against β-catenin and β-actin overnight at 4˚C. Membranes were then washed three times using Tris-buffered saline with 0.1% Tween-20 (TBST) for 10 min/wash. Subsequently, membranes were incubated with 1:2,500 goat-anti-rabbit and goat-anti-rat immunoglobulin G for 1 h at the room temperature. TBST was used to wash the membranes three times for 10 min/wash. Protein bands were visualized using a configured illuminant solution. Proteins were then analyzed using ImageJ 2.0 (National Institutes of Health, Bethesda, MD, USA) to assess the differential expression of β-catenin.

Detection of cell proliferation using MTT. The 3 cell groups in the logarithmic phase were selected and processed as previously described. A total of 0.75x10^4/ml cells in suspension were inoculated onto a 96-well plate (200 μl/well). The 96-well plate was then incubated at 37˚C in 5% CO_2. Cells were removed and observed using an inverted phase contrast microscope (magnification, x200). Cells were then cultured for a further 24 h at 37˚C and 5% CO_2. Cultivation was terminated after a 4 h incubation with 5 mg/ml MTT at room temperature for 4 h. Each experiment was repeated three times.

Detection of cell clone formation. The 3 cell groups exhibiting logarithmic phase growth were selected and processed as aforementioned. A total of 1x10^3/ml cells were inoculated onto a 6-cm sterile petri-dish and 200 μl suspension was added. Each group treatment was then repeated twice. Petri-dishes were incubated at 37˚C in 5% CO_2 for 14 days. Following visible clone growth, culture medium was removed and the remaining cells were washed three times with PBS. Cells were then stained with crystal violet for 10 min. Following staining, dishes were placed on a transparent grid

and the number of clone cells was counted using a WTDS-1 inverted microscope (magnification, x400; Shenzhen weite photoelectric instrument sales department, Shenzhen, China). Clone formation rate was calculated using the formula: Clone formation rate=number of clones formed/Inoculation cell number x 100.

Transwell invasion and migration assays. The 3 cell groups in the logarithmic growth phase were selected and processed as aforementioned. A total 1x10^5/ml of cells in suspension were plated in the upper chambers of Transwell plates in DMEM containing 10% bovine serum albumin (BSA; BioWit Technologies Ltd., Shenzhen, China). Transwell membranes were pre-coated with Matrigel and diluted with 10% BSA in a ratio of 1:6. A total 50 μl solution was added to each well of the upper chamber. Each cell group was allocated a total of 4 chambers, placed in a 24-well plate and incubated at 37˚C with 5% CO_2 for 1 h to solidify the gel. Following incubation, chambers were removed and 50 μl DMEM with 10% BSA was added. This was incubated for a second time at 37˚C for 30 min. The lower chambers of the Transwell plate were plated with 500 μl DMEM containing 10% BSA. Matrigel-coated chambers were subsequently placed into the 24-well plate and incubated for 24 h. Following incubation, migratory cells were stained with 0.5% crystal violet at 37˚C for 20 min and counted in nine randomly-selected fields using an inverted microscope (magnification, x400; Shenzhen Weite Photoelectric Instrument Sales Department). An average cell count was then calculated. A Transwell migration experiment All incubation and staining conditions used during this procedure were the same as those used for the Transwell invasion. The steps of this experiment were the same as aforementioned Transwell invasion method, however, in this protocol, Matrigel was not used. A total of 5x10^5/ml cells were directly inoculated onto the upper chambers of the Transwell plate. Lower chambers were inoculated with DMEM with 10% BSA. Cell staining was performed following 8 h culture.

Detection of cell apoptosis. The 3 groups of cells in the logarithmic phase were selected. Following conventional processing, an appropriate quantity of 1x Buffer A was used to wash cells, once. Following treatment, cells were centrifuged at a speed of 7,300 x g for 5 min at room temperature so that the transfer buffer (Beijing Transgen Biotech Co., Ltd.). Cells were stored in a refrigerator for 16 h at 20˚C Samples were then removed and centrifuged at a speed of 448 x g and a temperature of 37˚C for 5 min. Following centrifugation, ethyl alcohol was removed and cells were re-suspended in 500 μl Buffer A. RNaseA was then added to adjust cell concentration to 0.25 mg/ml and cells were incubated at 37˚C for 30 min. A total of 5 ml 0.5% crystal violet temperature according to the kit requirement (apoptosis PI staining kit; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was then added and left for 30 min at 22˚C. Flow cytometry (Flowjo software; version 10.4.2; BD Biosciences, Franklin Lakes, NJ, USA) was used to detect cell apoptosis rate, which was analyzed at a wavelength of 488 nm. A flow cytometer was used to analyze cell DNA content. The presence of a sub G1 peak indicated that a cell was apoptotic.

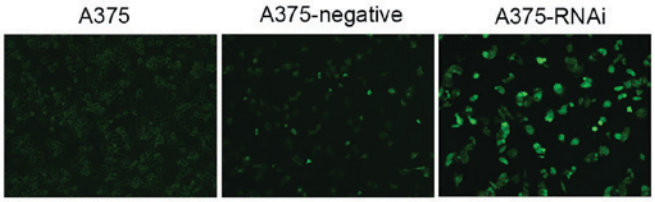

Figure 1. Cell morphology observed using an inverted microscope (magnification, x200). A375-RNAi cells and A375-negative cells exhibited a strong green fluorescence; wheras A375 cells did not. siRNA, short interfering RNA.

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was utilized to analyze the results of this study. One-way analysis of variance was performed to assess differences among groups and a student Newman-Keuls test was performed if variance was consistent. If discrepancies between groups were identified, the Games-Howell variance method was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cellular morphology. Strengthened adherence and an increase in cell number was observed in A375-RNAi cells compared with A375 and A375-negative cells (Fig. 1). The doubling time of A375-negative and A375-RNAi cells was also prolonged. The size and morphology of uninfected control cells was normal: The cytoplasm was evenly distributed, good refractivity was observed and cell reproduction was fast. No significant differences were identified between the morphologies of A375-negative and A375-RNAi. There was no significant difference between A375-negative and A375 cells.

Western blot analysis and β-catenin expression. As presented in Fig. 2, β-catenin expression in A375-RNAi cells decreased markedly compared with that of A375 cells indicating that the lentiviral vector-mediated siRNA silenced β-catenin expression. No marked differences between the expression of β-catenin in A375-negative cells and A375 cells were identified. The lentiviral empty vector therefore did not significa1ntly influence the expression of β-catenin.

Cell proliferation. The results of the MTT assay (Table I) demonstrated that the proliferation of A375-RNAi cells was significantly inhibited at all time points, compared with A375-negative cells ($P<0.05$; Table I).

Invasion and migration of A375 cells. The invasive abilities of A375-RNAi cells in the experiment group were significantly inhibited *in vitro* compared with A375-negative cells ($P<0.05$; Table II; Fig. 3A). The invasiveness of A375 and A375-negative cells was similar. The migration ability of A375-RNAi cells in the experiment group was also significantly inhibited compared with A375-negative cells ($P<0.05$; Table III; Fig. 3B). A375 cells in the blank control group and A375-negative cells in the negative control group exhibited similar migration abilities *in vitro*.

Table I. Influence of lentiviral vector-mediated siRNA on A375 cell proliferation.

Time (h)	A375	A375-negative	A375-RNAi
24	0.215±0.082	0.214±0.085	0.184±0.077[a]
48	0.371±0.025	0.356±0.019	0.292±0.082[b]
72	0.593±0.039	0.572±0.027	0.499±0.033[b]

[a]P<0.05 in A375 and A375-RNAi vs. A375-negative cells. A375-RNAi cell *in vitro* proliferation was significantly inhibited compared with the blank control group (corresponding time points; [b]P<0.05). siRNA, small interfering RNA; A375-RNAi, A375-RNA interference. Method: compared A375 with A375-negative; compared A375 with A375-RNAi, P<0.05; compared A375-negative with A375-RNAi, P<0.05.

Table II. Influence of lentiviral vector-mediated siRNA on A375 cell invasion.

Group	A375	A375-negative	A375-RNAi
Number of penetrating cells	44.54±3.47	41.15±3.64	22.58±2.20[a]

[a]P<0.05 vs. A375-negative cells. siRNA, small interfering RNA; A375-RNAi, A375-RNA interference.

Table III. Influence of lentiviral vector-mediated siRNA on A375 cell migration.

Group	A375	A375-negative	A375-RNAi
Number of penetrating cells	71.13±5.46	70.72±4.51	53.33±49.15[a]

[a]P<0.05 vs. A375-negative cells. siRNA, small interfering RNA; A375-RNAi, A375-RNA interference.

Table IV. Influence of lentiviral vector-mediated siRNA on A375 cell apoptosis.

Group	A375	A375-negative	A375-RNAi
Sub G_1 phase (%)	3.13±0.46	4.02±0.59	20.57±3.26[a]

[a]P<0.05 vs. A375-negative cells. siRNA, small interfering RNA; A375-RNAi, A375-RNA interference.

Cellular apoptosis. As presented in Table IV, the apoptosis rate of A375-negative cells was 4.02±0.59%. However, the apoptosis rate of A375-RNAi cells in the experiment group increased significantly to 20.57±3.26% (P<0.05). The cell apoptosis rate of A375 cells was 3.13±0.46%, similar to that of A375 cells.

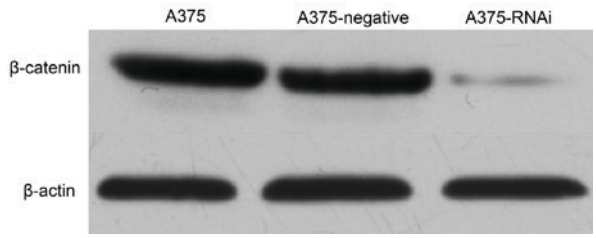

Figure 2. Cellular expression influence of lentiviral vector-mediated siRNA on the expression of β-catenin and β-actin in A375 cells. siRNA, small interfering RNA.

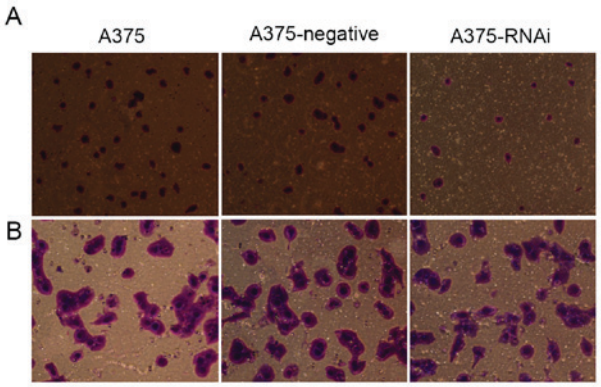

Figure 3. Influence of lentiviral vector-mediated siRNA on A375 cell (A) invasion and (B) migration *in vitro*. siRNA, small interfering RNAm (magnification, x200).

Discussion

Excessive and fast cell proliferation is one of the primary causes of high-grade MM malignancy. Katiyar and Vaid (15) and Widlund *et al* (16) demonstrated that activation of the Wnt/β-catenin signaling pathway increased MM cell clone formation and proliferation *in vitro*. Previous studies have also demonstrated that the upregulation of Dickhopf-related protein 1 (DKK1) significantly downregulates β-catenin, thus reversing the inhibitory effect of DKK1 on A375 cell proliferation (17,18). In MM tissue, DKK1 expression is significantly lower than in normal human epidermal tissue (19). In addition, the expression of β-catenin in A375 cells is significantly higher than that of epidermal cells (20). Therefore, it was hypothesized that the downregulation of β-catenin may inhibit cell proliferation and clone formation. However, other studies (21-23) have demonstrated that β-catenin is found in the majority of benign melanocytic nevi and that a decrease in β-catenin content may cause the development of MM. Cell cultures performed in this study revealed that A375 cells grow actively. The results of the current study also demonstrated that the downregulation of β-catenin inhibits A375 cell proliferation and clone formation. However, the specific mechanism that underlies this process requires further study.

Another cause of high grade malignancy is the invasive and migratory abilities of MM tumor cells. Tobias *et al* (24) demonstrated that the transcriptional activity of β-catenin increases gradually as MM develops. Previous studies have revealed that β-catenin serves a vital role in two

primary processes: β-catenin/E-cadherin mediated cell adhesion and the transcription and regulation of classical Wnt/β-catenin signaling in the cell nucleus (25,26). As MM develops, the expression of E-cadherin gradually decreases, which inhibits the formation of β-catenin/E-cadherin cell adhesion compounds, resulting in cell invasion and migration (27). However, previous studies have demonstrated that the upregulation of β-catenin may improve the prognosis of patients with MM (28,29). Murine studies have demonstrated that the downregulation of β-catenin may accelerate the metastasis of MM. Invasion and migration experiments in the present study demonstrated that β-catenin silencing significantly decreases the invasion and migration of A375-RNAi cells, compared with that of A375 cells *in vitro*. However, no significant differences in the migration and invasion between A375-negative and A375 cells were identified. In benign melanocytic nevi and pre-invasive MM cells, β-catenin is primarily located in the cell membrane and in E-cadherin cell adhesion compounds. This serves to increase intercellular adhesion and limit cell invasion (30). As MM increases in malignant grade due to the loss of casein kinase 1α activity, the expression and transcriptional activity of intracellular β-catenin increases (31). Vaid *et al* (32) revealed that the downregulation of β-catenin effectively inhibits the invasion and migration of A375 cells. This may be due to the downregulation of β-catenin decreasing the formation of E-cadherin cell adhesion compounds, thus resulting in easily detachable cells that break away from the tumor. It has also been demonstrated that the downregulation of β-catenin in A375 cells effectively inhibits cell invasion (33).

Cellular apoptosis is primary regulated by the B cell lymphoma 2 (Bcl-2) protein family. Bcl-2 can inhibit apoptosis by attenuating the activation of Bax and Bak. It has been demonstrated that β-catenin may regulate the expression of Bax and Bcl-2 (34). c-Myc is one of the primary target genes of β-catenin, which influences cell proliferation and apoptosis by activating or inhibiting genetic transcription. c-Myc may become overexpressed and induce apoptosis when there is a lack of cell growth and activation due to nutrient deficiencies (35,36). In the present study, the apoptosis rate of A375-RNAi cells was significantly higher than that of A375 cells. However, no significant differences in the apoptosis rates of A375-negative cells and A375 cells was identified. Schittek *et al* (37) assessed multiple MM cells and the apoptosis of MM cells and demonstrated that β-catenin regulates the apoptosis of MM cells. This may be due to a decrease in the expression of Bcl-2 and c-Myc and an increase of Bax, (mediated by the inhibition of β-catenin), reversing the inhibition of apoptosis, thus accelerating apoptosis.

In conclusion, the inhibition of β-catenin expression or activity may inhibit the proliferation, invasion and migration of cells and may induce the downregulation of anti-apoptosis genes, thus accelerating apoptosis. The results of the present study also indicate that β-catenin may be an effective target for the treatment of patients with MM.

Competing interests

The authors declare that they have no competing interests.

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