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Adenoviral vector expressing short hairpin RNA targeting Wnt2B has an effective antitumour activity against *Wnt2B2*-overexpressing tumours

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ABSTRACT

Background: The Wnt family encodes multi-functional signalling glycoproteins regulating various normal and pathological processes including tumourigenesis. Wnt2B overexpression is thought to affect tumour progression through the activation of the canonical Wnt pathway.

Method: Experimental studies were conducted using a Wnt2B-inhibiting vector to establish gene therapy against *Wnt2B2*-overexpressing tumours. A replication-deficient recombinant adenoviral vector expressing short hairpin RNA targeting Wnt2B (Ad-shWnt2B) was constructed. Three *Wnt2B2*-overexpressing human tumour cells, including A549 cells, Hela cells and PANC1 cells, were used. Thereafter, cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Next, a human tumour xenograft model in nude mice was prepared by subcutaneously implanting tumours derived from A549 cells. Ad-shWnt2B was administered via intratumoural injection every 4 days.

Results: First, immunohistochemical studies revealed that high levels of Wnt2B expression appeared in proliferative normal tissues and many human tumour tissues. Furthermore, the *Wnt2B2* gene expression was associated with c-*Myc* and *survivin* expressions in human lung cancer. Transduction with Ad-shWnt2B effectively downregulated the *Wnt2B2* expression in all the three *Wnt2B2*-overexpressing tumour cells ($p < 0.0001$). The transduction with Ad-shWnt2B significantly reduced the percentage of viable cells in all the *Wnt2B2*-overexpressing tumour cells ($p < 0.005$). In addition, transduction with Ad-shWnt2B significantly downregulated c-*Myc* and *survivin* in A549 cells ($p < 0.005$). Furthermore, the treatment with Ad-shWnt2B exerted a significant antitumour effect against the *Wnt2B2*-overexpressing A549 xenografts by inducing apoptosis ($p < 0.01$).

Conclusions: Cancer gene therapy using an adenoviral vector expressing short hairpin RNA (shRNA) against Wnt2B was, therefore, found to have a strong antitumour effect against *Wnt2B2*-overexpressing tumours.

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1. Introduction

The Wnt gene family encodes multi-functional signalling glycoproteins regulating various normal and pathological processes.[1–3] Members of the Wnt family involved in the canonical pathway can induce various tumour-associated targets,[4,5] such as c-Myc[6] and survivin.[7,8] c-Myc is a transcription factor that plays a role in cell proliferation.[9,10] Survivin is also predicted to promote tumourigenesis by regulating not only the apoptotic inhibition[11] but also the tumour cell proliferation.[12–14] Clinical studies have demonstrated the expressions of these Wnts to be frequently upregulated in various human cancers.[15–18] Furthermore, we found that the transfection of the metastatic suppressor gene *MRP-1/CD9* could downregulate several Wnt members, including *Wnt2B*, in tumour cells.[19] Therefore, these Wnt members could be candidates of molecular targets for cancer therapy.[20]

The successful application of RNAi in cancer gene therapy depends on the efficient delivery of small interfering RNA (siRNA) into the cells.[21–23] Stable siRNA molecules can be produced via short hairpin RNA (shRNA) expressed under the control of the RNA polymerase III-dependent promoter.[24,25] Furthermore, adenoviral vectors have been widely used for the expression of transgenes not only in experimental conditions[26,27] but also in the clinical setting.[28] Therefore, to establish a new treatment for tumours with an overexpression of Wnt2B2, a major transcript of the human Wnt2B gene,[29] we constructed an adenoviral vector expressing shRNA targeting Wnt2B. Consequently, this vector had an effective antitumour activity against *Wnt2B2*-overexpressing tumours both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell lines

Eight human tumour cells, including lung carcinoma A549 cells and MAC10 cells, head and neck carcinoma Hela cells, colorectal carcinoma DLD-1 cells and HT-29 cells, pancreatic carcinoma BxPC3 cells and PANC1 cells and fibrosarcoma HT1080 cells, were used.

2.2. Design and transfection of siRNA

Three siRNA oligonucleotides targeting Wnt2B were designed using the siRNA Design Support System (Takara Biomedicals, Otsu, Japan). The sense strand sequences were: Wnt2B-siRNA1, 5′-CAACUCUCCAGAUUACUGUTdT-3′; Wnt2B-siRNA2, 5′-CAAGGAAUGCAGAAAUACUTdT-3′; and Wnt2B-siRNA3, 5′-CACGAGUGAUCUGUGACAATdT-3′. The siRNA transfection was performed in a total volume of 3 ml containing each siRNA (final concentration of 25 nM) and 25 μl of TransIT-TKO Transfection Reagents (Mirus, Madison, WI).

2.3. Construction of adenoviral vectors

The shRNA template [forward strand: 5′- Wnt2B-siRNA1 sense strand (CAACTCTCCAGATTACTGT) + loop (TAGTGCTC CTGGTTG) + Wnt2B-siRNA1 antisense strand (ACAGTAATCTGGAGAGTTG) + polymerase III terminator (TTTTTT)] was synthesised. To produce a plasmid vector expressing shRNA (plasmid–shWnt2B), this shRNA template was cloned into a pBAsi-hU6 plasmid vector (Takara). Thereafter, an adenoviral vector was constructed using an Adenovirus Expression Vector Kit (Takara). The insert with the human RNA polymerase III-dependent U6 promoter and shRNA template was produced from plasmid–shWnt2B using restriction enzyme digestion by EcoRV and it was then ligated into a pAxcwit cosmid vector. A replication-deficient recombinant adenoviral vector expressing shRNA targeting Wnt2B under the control of the human U6 promoter (Ad-shWnt2B) was constructed using the COS-TPC method.[30] A control adenoviral vector expressing shRNA against the scrambled sequence of Wnt2B–siRNA1 was also constructed (Ad-scramble). Constructed adenoviral vectors were amplified in 293 HEK cells and purified by CsCl ultracentrifugation.

2.4. Quantitative RT-PCR

Total cellular RNA was extracted using the acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed using a cDNA synthesis kit (Amersham Bioscience, Piscataway, NJ). TaqMan real-time quantitative PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probes were from the Assays-on Demand Gene Expression Assay (*Wnt2B2* assay ID Hs00257131_m1; c-*Myc* assay ID Hs00153408_m1, Applied Biosystems). Each sample was run in triplicate. The comparative threshold cycle method was used to calculate the gene expression in each sample relative to the value in the A549 cells, using *GAPDH* (assay ID Hs99999905_m1) as a control for normalisation among samples. RNA samples isolated from three independent experiments were evaluated.

Because real-time PCR is not an appropriate method to discriminate between *survivin* (wild-type *survivin*) and other splice variants with different biological functions,[31] we carried out quantitative RT-PCR assays using densitometric analyses of gel electrophoresis to evaluate the *survivin* gene expressions, as reported previously.[32]

2.5. Immunohistochemistry

Immunohistochemistry was performed using Tissue Array Slide (Super Bio Chips, Seoul, Korea) and 95 non-small cell lung cancers (NSCLCs) from consecutive patients who underwent surgery at Kagawa University from January 2003 to December 2004. This study was approved by the institutional review board and signed informed consent was obtained from each patient. After deparaffinised and rehydrated, sections of paraffin-embedded tissues were heated in a microwave for 10 min. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, sections were treated for 2 h with 5% bovine serum albumin. Sections were incubated overnight with the primary specific antibodies detecting Wnt2B (LS-C31588; LifeSpan BioSciences,

Seattle, WA, 1.5 μg/ml) and the Ki-67 antigen (MIB-1; DAKO, Glostrup, Denmark, 1:40). After incubated for 1 h with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), sections were incubated with the avidin–biotin–peroxidase complex (Vector Laboratories) for 1 h. Antibody binding was visualised with 3,3′-diaminobenzidine tetrahydrochloride. Lastly, sections were counterstained with Mayer's haematoxylin. The percentage of carcinoma cells with positive staining for Ki-67 was scored as the Ki-67 proliferation index.

2.6. Detection of apoptosis

Apoptotic cells were detected using the In Situ Apoptosis Detection Kit (Takara). After deparaffinised and rehydrated, sections were treated with 20 μg/ml Proteinase K for 15 min. After quenching the endogenous peroxidase activity with 3% H_2O_2 for 5 min, the sections were incubated with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) reaction mixture, including terminal deoxynucleotidyl transferase (TdT), for 90 min at 37 °C. Next, the sections were incubated with anti-FITC horseradish peroxidase conjugate for 30 min at 37 °C. Staining was developed using 3,3′-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly counterstained with Mayer's haematoxylin. The apoptotic index was defined as the number of apoptotic cells per 1000 tumour cells.

2.7. Western blot analysis

Cells were harvested and resuspended in lysis buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 4 M urea). Protein samples (50 μg) were each diluted into a 20 μl solution of lysis buffer and 5% 2-mercaptoethanol and heated at 95 °C for 5 min. The protein extracts were separated by 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose membrane and then blocked in a blocking solution (5% dry milk and 0.2% Tween 20 in phosphate buffered saline (PBS)) for 1 h. The membranes were incubated overnight with the primary specific antibodies detecting Wnt2B (LS-C31588; LifeSpan BioSciences, 1.5 μg/ml), c-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA, 1:100), and survivin (sc17779, Santa Cruz, 1:100). The membranes were then incubated with HRP-labelled secondary antibodies for 1 h. The proteins were visualised on enhanced chemiluminescence film. Finally, the blots were reprobed using a mouse anti-human actin mAb (C-2; Santa Cruz, 1:1000) to ensure equal loading and transfer of proteins. Each experiment was repeated three times with consistent results.

2.8. Cell viability assay

Tumour cells were seeded in 96-well plates at concentration of 2000 cells/well. The cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a Cell Proliferation Kit I (Roche, Mannheim, Germany). Tumour cells were incubated with 10 μl of MTT labelling reagent for 4 h, and then were incubated with 100 μl of solubilisation solution overnight. Finally, the cell viability in each well was measured in terms of optical density at a wavelength of 570 nm, with 750 nm for reference. Each cell viability assay was performed in triplicate and repeated three times.

2.9. Flow cytometric analysis

Cells were fixed in 70% ethanol at 4 °C overnight. Fixed samples were treated with 250 mg/ml RNase A at 37 °C for 1 h and resuspended in 60 μg/ml propidium iodide for 30 min. The stained cells were analysed in a Cytomics FC500 (Beckman Coulter, Tokyo, Japan). Apoptotic cells were represented by the fraction of cells in the sub-G1 phase. Each experiment was repeated three times with consistent results.

2.10. Immunocytochemistry

For double staining of a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and 4′-6-diamidino-2-phenylindole (DAPI, Molecular Probes, OR, USA) staining, cells grown on dishes were fixed for 10 min in 3.7% paraformaldehyde in PBS. The TUNEL assay was first performed with the Apop Tag Red Plus In Situ Apoptosis Detection Kit (Intergen Company, NY, USA). The apoptotic cells were visualised with the Rhodamine-conjugated antibody. The cells were then incubated for 20 min in DAPI solution (400 nM DAPI and 1% albumin in PBS). The fluorescent signals were visualised using a confocal microscope (Bio-Rad Radiance 2100, Bio-Rad). For each determination, the photographs of nine randomly selected fields were analysed. Each experiment was repeated three times with consistent results.

2.11. Human tumour xenograft model in nude mice

Tumour xenografts were prepared by implanting approximately 8 mm^3 fragments of tumours derived from A549 cells subcutaneously into the back of 6-week-old male nude mice. When the tumour volume reached approximately 100 mm^3, the mice were randomly divided into three groups (eight mice/group): groups treated with Ad-shWnt2B and Ad-scramble, and control group treated with PBS. Intratumoural injection with adenoviral vectors (at 2×10^9 PFU, respectively) or 0.5 ml of PBS was performed every 4 days. Tumour growth was monitored every 3 days for 30 days by measuring tumour size using a caliper. The tumour volume was calculated by the following formula: tumour volume = $(\text{length}) \times (\text{width})^2 \times 0.5$. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals from Kagawa University.

3. Results

3.1. Wnt2B expression in normal and tumour tissues

Wnt2B protein expression in normal and tumour tissues was evaluated by immunohistochemistry (Fig. 1A). Regarding normal tissues, various proliferative tissues such as the basal layer of skin and placenta had high levels of Wnt2B expression. However, many differentiated tissues, such as the lung alveolar epithelium, had either negative or low levels of Wnt2B expression. In contrast, many human tumours, including lung cancer, had high levels of Wnt2B expression.

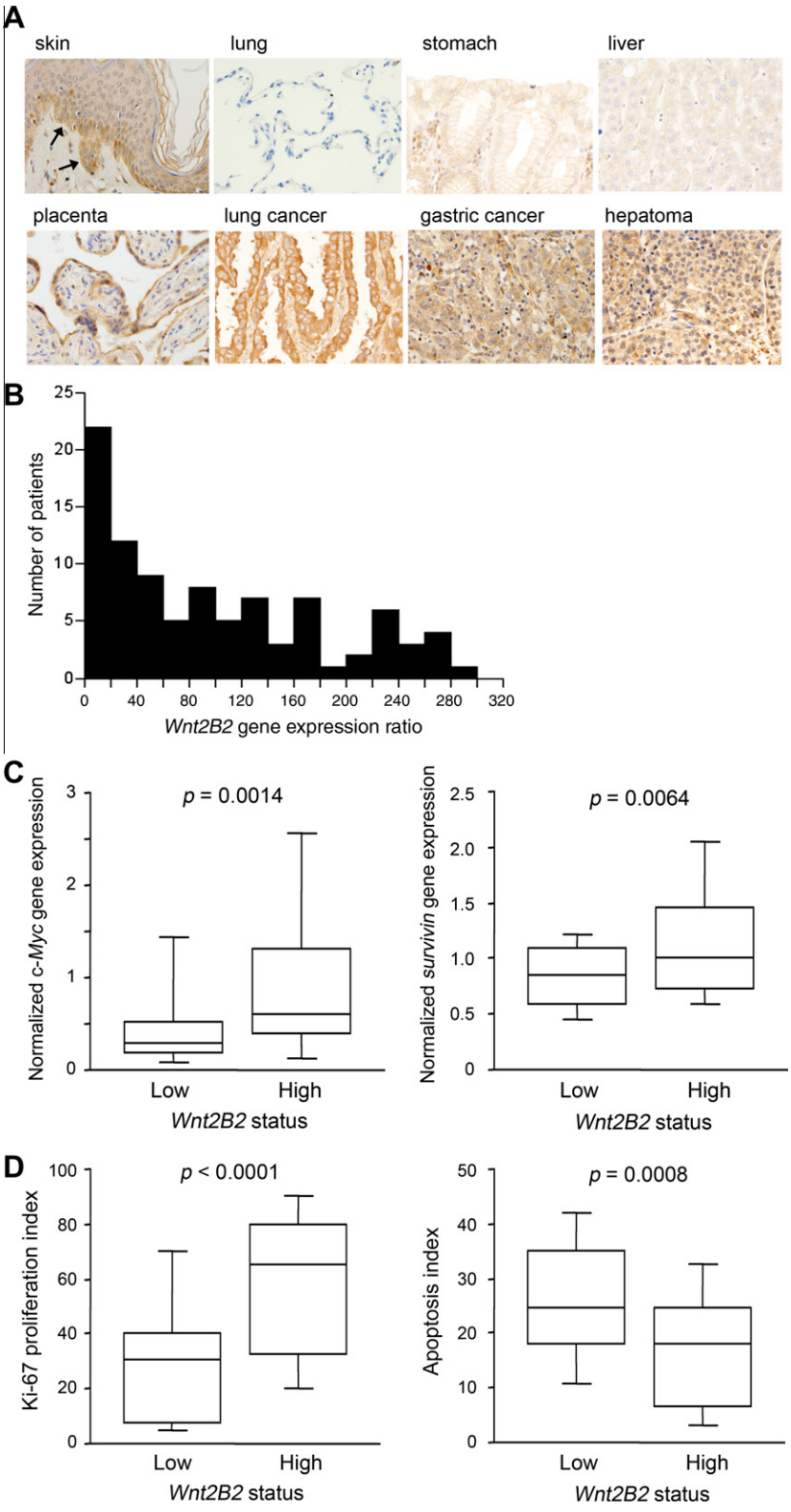

Fig. 1 – (A) Wnt2B protein expressions in normal and tumour tissues. Arrow, basal layer of skin with high levels of Wnt2B expression. (B) *Wnt2B2* gene expressions in 95 non-small cell lung cancers. (C) c-Myc and *survivin* gene expressions in relation to the *Wnt2B2* status in 95 non-small cell lung cancers. (D) Ki-67 proliferation index and apoptotic index in relation to the *Wnt2B2* status in 95 non-small cell lung cancers.

3.2. Clinical significance of the Wnt2B2 expression in NSCLCs

Next, the clinical significance of the *Wnt2B2* gene expression in NSCLCs was investigated. The *Wnt2B2* gene expression ratio varied greatly among the 95 NSCLCs (median ± SD, 0.79 ± 0.73; Fig. 1B). These NSCLCs were classified into two groups, 47 *Wnt2B2*-high tumours and 48 *Wnt2B2*-low tumours, using a median value as a cut-off line. Regarding Wnt-target genes, c-*Myc* and *survivin* gene expressions were significantly higher in *Wnt2B2*-high tumours than in *Wnt2B2*-low tumours ($p = 0.0014$ and $p = 0.0064$, respectively, Fig. 1C). Furthermore, the Ki-67 proliferation index was significantly higher in *Wnt2B2*-high tumours than in *Wnt2B2*-low tumours ($p < 0.0001$, Fig. 1D). The apoptotic index was significantly lower in *Wnt2B2*-high tumours than in *Wnt2B2*-low tumours ($p = 0.0008$, Fig. 1D).

3.3. Selection of Wnt2B2-overexpressing tumour cells

The normalised *Wnt2B2* expression ratio was evaluated in eight human tumour cells (Fig. 2A). As a result, three tumour cells, A549 cells, Hela cells and PANC1 cells, were selected as *Wnt2B2*-overexpressing tumour cells.

3.4. Identification of specific sequence of siRNA targeting Wnt2B

Three chemically-synthesised siRNAs designed from different sequences of the Wnt2B were investigated. Seventy-two hours after the siRNA transfection into A549 cells, Wnt2B–siRNA1 caused the strongest downregulation of the *Wnt2B2* gene expression (90.9 ± 4.4%; Fig. 2B) and the Wnt2B protein expression (Fig. 2C). Therefore, Wnt2B–siRNA1 was selected as the specific target site to construct a Wnt2B-inhibiting adenoviral vector (Ad-shWnt2B).

3.5. Ad-shWnt2B efficiently downregulates the Wnt2B2 expression

Three *Wnt2B2*-overexpressing tumour cells, A549 cells, Hela cells and PANC1 cells, were transduced with Ad-shWnt2B at a multiplicity of infection (MOI, PFU/cell) of 5, 10, 20 (Fig. 3A–C). Ad-shWnt2B effectively knocked down the *Wnt2B2* gene expression in all the three *Wnt2B2*-overexpressing tumour cells in a time- and dose-dependent manner ($p < 0.0001$ versus Ad-scramble, respectively). The downregulation of Wnt2B protein expression was also detected after transduction with Ad-shWnt2B in all the *Wnt2B2*-overexpressing tumour cells (Fig. 3D).

3.6. Ad-shWnt2B inhibits the growth of Wnt2B2-overexpressing tumour cells

Next, we investigated the inhibitory effect of Ad-shWnt2B against *Wnt2B2*-overexpressing tumour cells (Fig. 4). The percentages of viable cells significantly decreased in all three *Wnt2B2*-overexpressing tumour cells transduced with Ad-shWnt2B ($p < 0.005$ versus Ad-scramble, respectively). Transduction with Ad-shWnt2B strongly reduced the percentage of viable cells in all the three *Wnt2B2*-overexpressing tumour cell lines in a time- and dose-dependent manner.

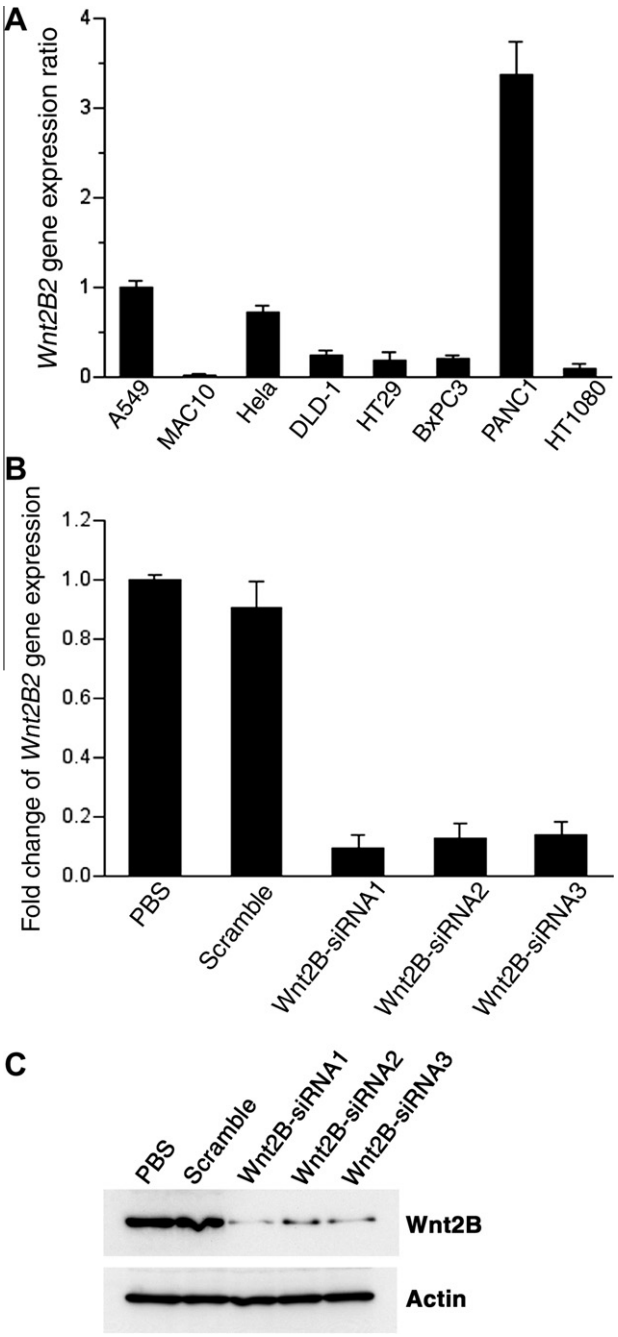

Fig. 2 – (A) *Wnt2B2* gene expressions in human tumour cell lines, (B) *Wnt2B2* gene expressions in lung carcinoma A549 cells after transfection of small interfering RNAs (siRNAs) targeting Wnt2B, (C) Wnt2B protein expressions in lung carcinoma A549 cells after transfection of siRNAs targeting Wnt2B. One of three experiments with similar results is shown. Scramble, scrambled oligonucleotides as a negative control against Wnt2B–siRNA1.

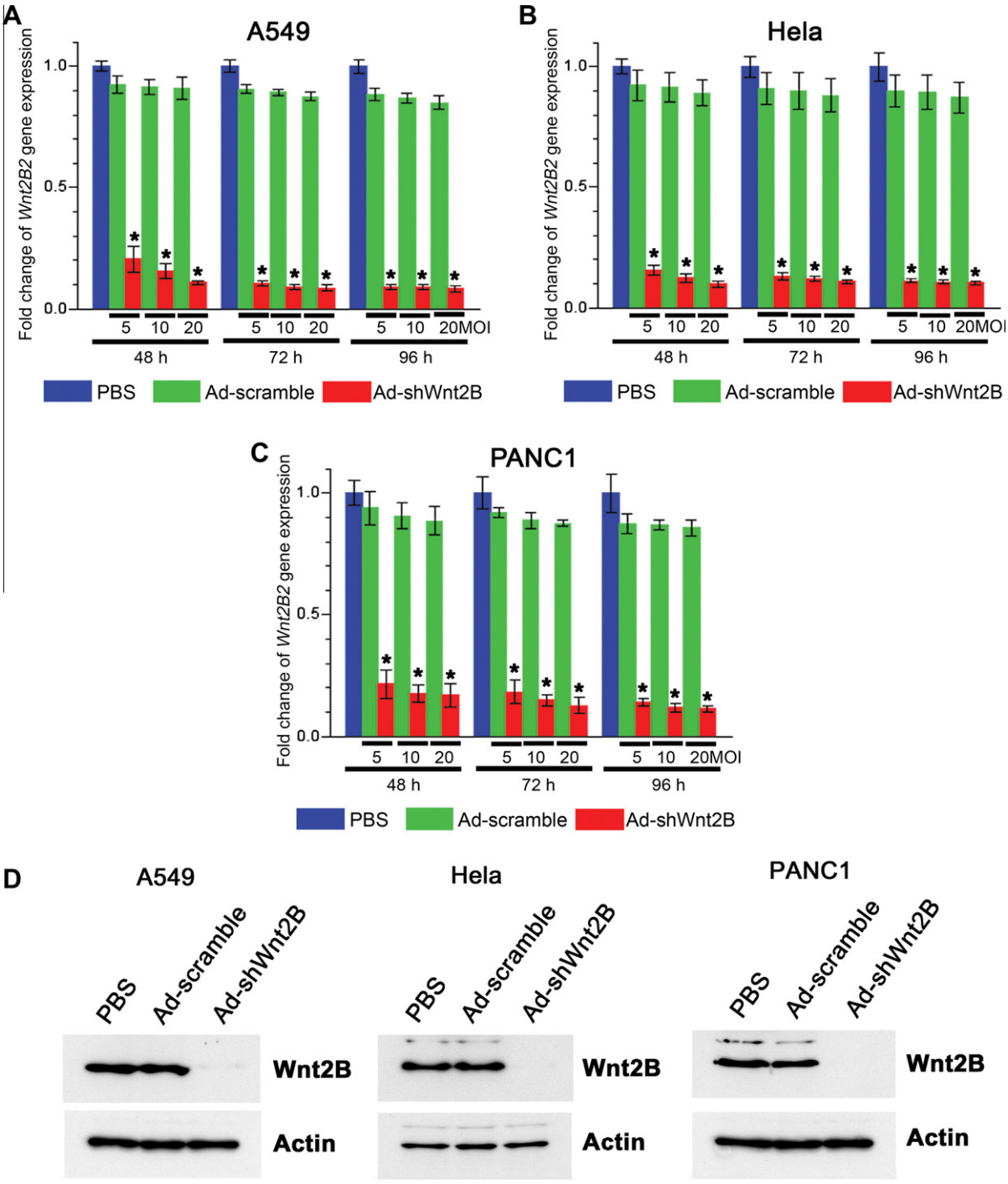

Fig. 3 – *Wnt2B2* gene expressions and Wnt2B protein expressions in *Wnt2B2*-overexpressing human tumour cells after transduction with adenoviral vectors. (A) *Wnt2B2* gene expressions in lung carcinoma A549 cells, (B) *Wnt2B2* gene expressions in head and neck carcinoma Hela cells, (C) *Wnt2B2* gene expressions in pancreatic carcinoma PANC1 cells, and (D) Wnt2B protein expressions in three *Wnt2B2*-overexpressing human tumour cells. Western blot analyses were performed at 72 h after transduction with adenoviral vectors at a multiplicity of infection MOI of 20 and one of three experiments with similar results is shown. MOI, multiplicity of infection; *$p < 0.0001$ versus Ad-scramble treatment.

3.7. *Ad-shWnt2B downregulates c-Myc and survivin expressions in Wnt2B2-overexpressing A549 cells*

c-*Myc* and *survivin* gene expressions were evaluated in the A549 cells after transduction with Ad-shWnt2B. The transduction with Ad-shWnt2B significantly downregulated c-*Myc* and *survivin* gene expressions in the *Wnt2B2*-overexpressing A549 cells in a time- and dose-dependent manner ($p < 0.005$ versus Ad-scramble, respectively; Fig. 5A and B). Furthermore, the transduction with Ad-shWnt2B

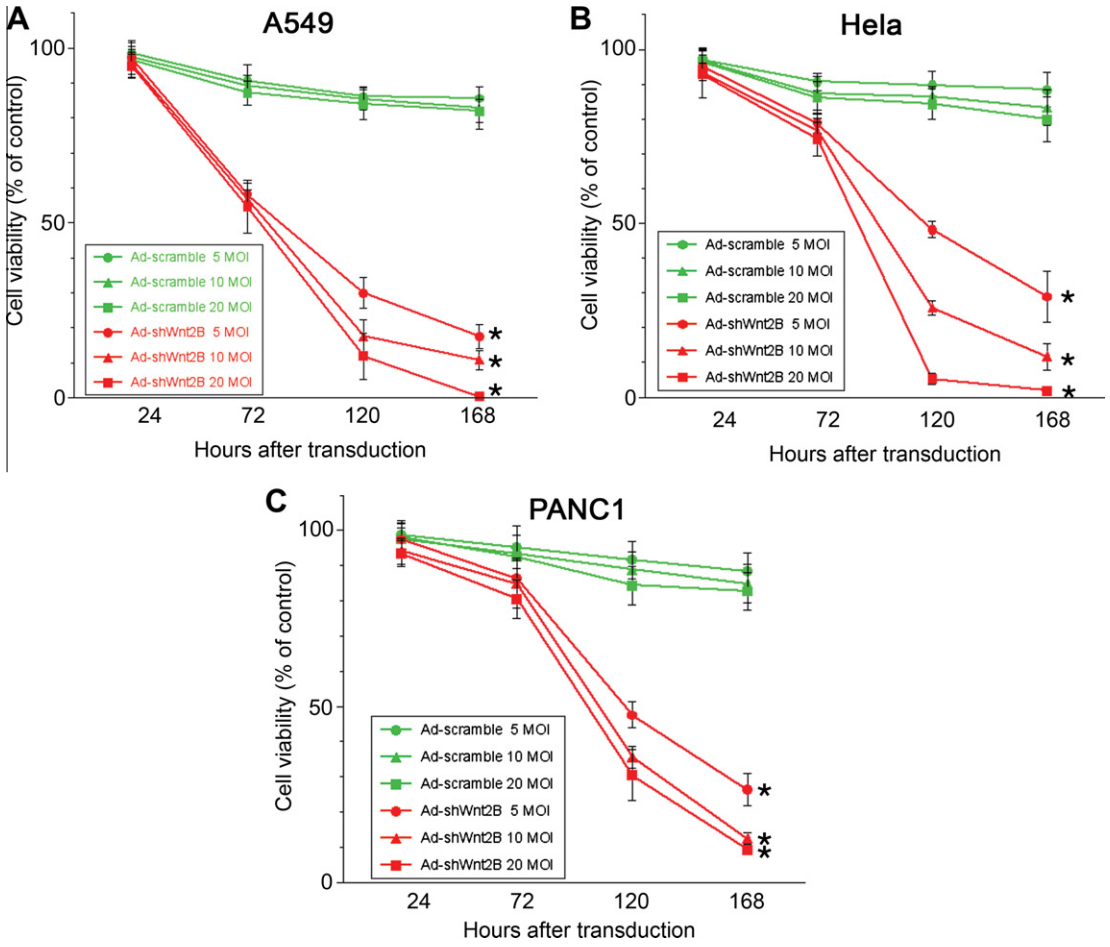

Fig. 4 – Cell viability evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after transduction with adenoviral vectors, in comparison to phosphate buffered saline (PBS)-treated control cells. (A) Lung carcinoma A549 cells, (B) head and neck carcinoma Hela cells and (C) pancreatic carcinoma PANC1 cells. MOI, multiplicity of infection; $^{*}p < 0.005$ versus Ad-scramble treatment.

downregulated c-Myc and survivin protein expressions in the A549 cells (Fig. 5C).

3.8. *Ad-shWnt2B induces apoptosis in Wnt2B2-overexpressing A549 cells*

A flow cytometric analysis was performed using A549 cells at 96 h after transduction with Ad-shWnt2B. The apoptotic cells were 1.6% in PBS-treated control cells and 77.8% in Ad-shWnt2B-treated cells (Fig. 5D). In addition, immunocytochemistry evaluated at 96 h after transduction also demonstrated the percentage of apoptotic cells to be significantly higher in the Ad-shWnt2B transfected A549 cells in comparison to the Ad-scramble transfected A549 cells (64.7 ± 6.3% versus 1.6 ± 0.7%, $p < 0.001$; Fig. 5E). The transduction with Ad-shWnt2B strongly induced apoptosis in the *Wnt2B2*-overexpressing A549 cells.

3.9. *Antitumour activity of Ad-shWnt2B against Wnt2B2-overexpressing tumour xenografts*

A *Wnt2B2*-overexpressing A549 tumour xenograft model was prepared in nude mice. The tumour volumes at day 30 were 489 ± 65 mm^3 in the PBS-treated groups, 449 ± 72 mm^3 in the Ad-scramble-treated groups and 179 ± 28 mm^3 in the Ad-shWnt2B-treated group (Fig. 6A). The Ad-shWnt2B treatment significantly inhibited A549 xenografts in comparison to PBS treatment and Ad-scramble treatment ($p < 0.01$, respectively).

Wnt2B protein expressions in the tumours at day 30 were evaluated by immunohistochemistry (Fig. 6B). The intratumoural Wnt2B protein expression in tumours treated with Ad-shWnt2B was remarkably lower in comparison to that in PBS-treated tumours and that in Ad-scramble-treated tumours. In addition, apoptotic cells in the tumours at day 30 were also analysed by the TUNEL method (Fig. 6C). The apoptotic index in tumours treated with Ad-shWnt2B was significantly higher in comparison to that in PBS-treated tumours and that in Ad-scramble-treated tumours ($p < 0.0001$ and $p = 0.0005$, respectively; Fig. 6D).

4. Discussion

Wnt2B stimulates the canonical Wnt pathway.[17] The *Wnt2B* gene is located on human chromosome 1p13.2,30. Two splicing variants occur from human *Wnt2B* gene due to alternative promoters.[29,33] *Wnt2B1* (NM_004185) encodes a secreted-type

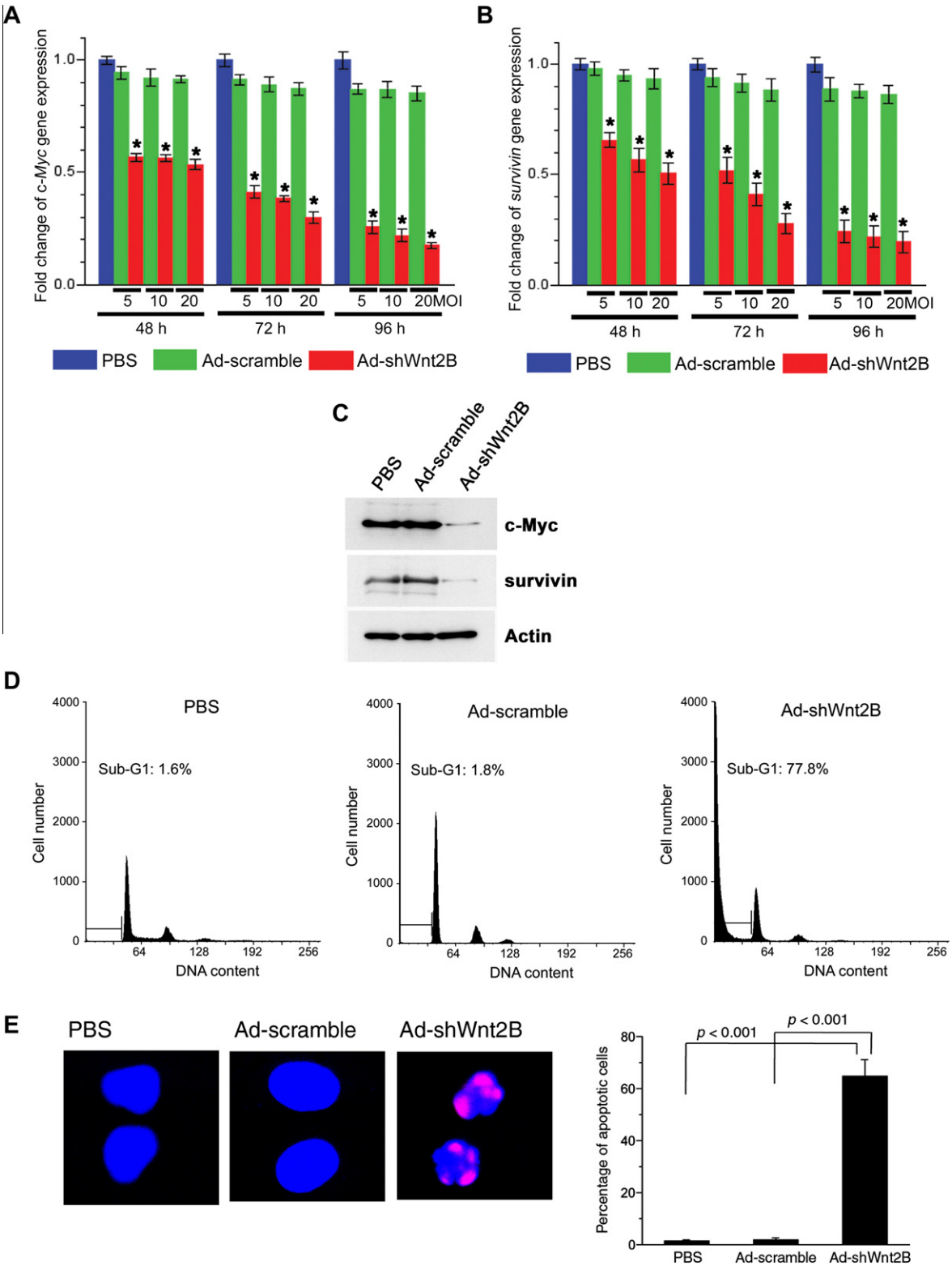

Fig. 5 – (A) c-*Myc* gene expression in *Wnt2B2*-overexpressing A549 cells after transduction with adenoviral vectors, (B) *survivin* gene expression in *Wnt2B2*-overexpressing A549 cells after transduction with adenoviral vectors, (C) c-Myc and survivin protein expressions in *Wnt2B2*-overexpressing A549 cells at 72 h after transduction with adenoviral vectors at a MOI of 20. One of three experiments with similar results is shown. (D) Flow cytometric analysis of propidium iodide-staining in *Wnt2B2*-overexpressing A549 cells 96 h after transduction with adenoviral vectors at a MOI of 20. Apoptotic cells were represented by the fraction of cells in the sub-G1 phase. One of three experiments with similar results is shown. (E) Immunocytochemistry in *Wnt2B2*-overexpressing A549 cells 96 h after transduction with adenoviral vectors at a MOI of 20. One of three experiments with similar results is shown. MOI, multiplicity of infection; $^{*}p < 0.005$ versus Ad-scramble treatment.

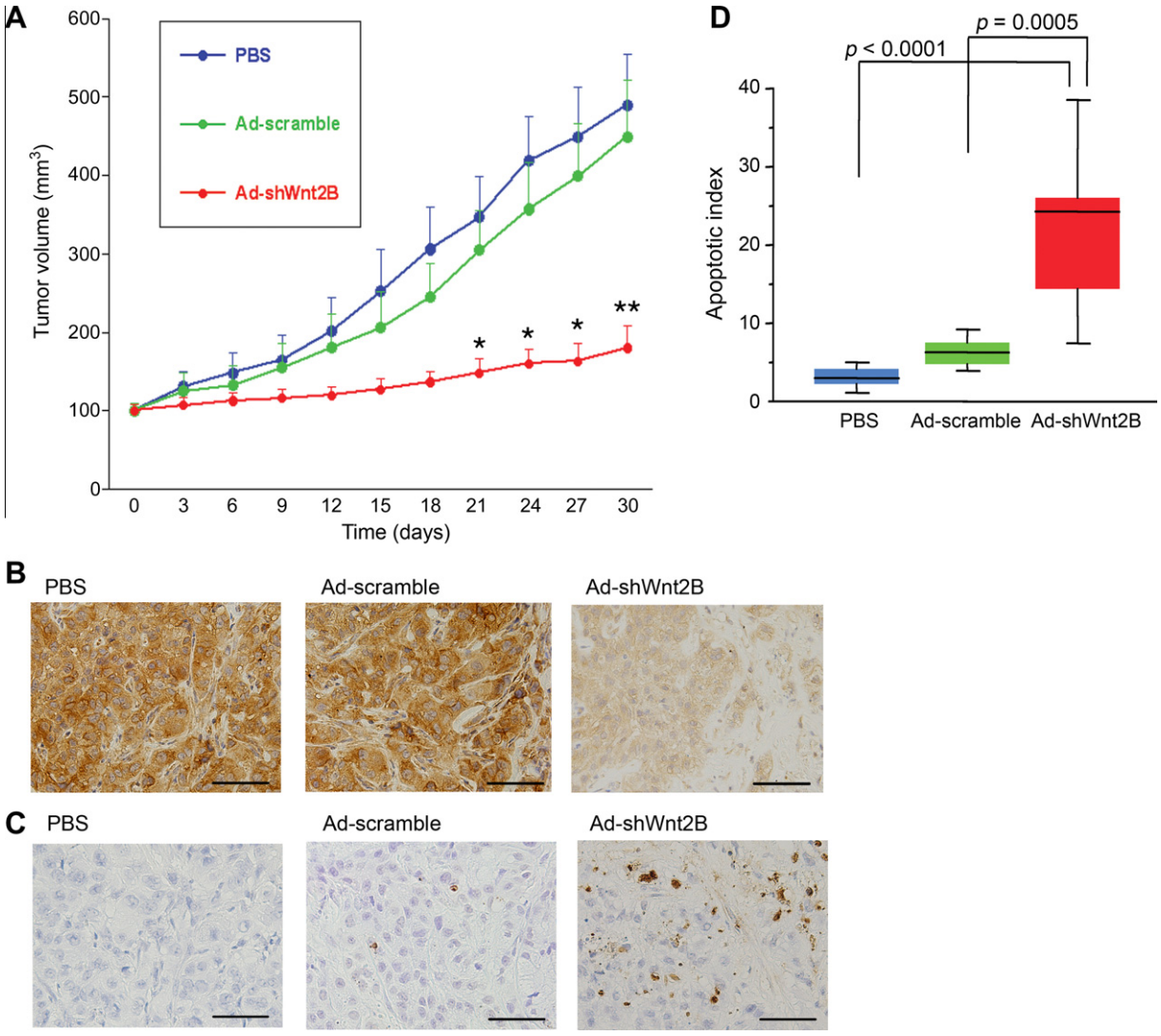

Fig. 6 – (A) Tumour volumes of *Wnt2B2*-overexpressing A549 xenografts in nude mice. $^{*}p < 0.05$ versus Ad-scramble treatment and versus PBS treatment; $^{**}p < 0.01$ versus Ad-scramble treatment and versus PBS treatment. (B) Immunohistochemical staining for Wnt2B of A549 xenografts in nude mice at 30 days after the initiation of treatment. Scale bar, 50 μm. (C) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining of A549 xenografts in nude mice at 30 days after the initiation of treatment. Scale bar, 50 μm. (D) Apoptotic index in *Wnt2B2*-overexpressing A549 xenografts in nude mice at 30 days after the initiation of treatment.

glycoprotein while *Wnt2B2* (NM_024494) encodes a transmembrane-type glycoprotein. Among them, *Wnt2B2* is the evolutionarily conserved major transcript of human Wnt2B, and its overexpression frequently occurs in various human cancers.[17,18,34] Therefore, the Wnt2B may be considered to affect tumourigenesis.

First, the present study evaluated the Wnt2B expression in various normal and tumour tissues. High levels of Wnt2B expression were observed in proliferative normal tissues and various human tumours. In contrast, many differentiated normal tissues had negative or low levels of Wnt2B expression.

Next, the present clinical study in NSCLC revealed that the *Wnt2B2* expression was associated with c-*Myc* and *survivin* expressions. As a result, the tumour proliferation rate was significantly higher in *Wnt2B2*-high tumours than in *Wnt2B2*-low tumours, and the apoptotic index was significantly lower in *Wnt2B2*-high tumours than in *Wnt2B2*-low tumours. Therefore, *Wnt2B2* overexpression may affect tumour progression, thus resulting in the production of more aggressive malignant tumours.

We also investigated the clinical significance of the *Wnt2* expression in NSCLCs, in our pilot study. However, the *Wnt2* expression was not associated with c-*Myc* or *survivin* gene expression, the Ki-67 proliferation index or the apoptotic index (data not shown). Therefore, we considered that the Wnt2B-inhibiting therapy could be a more important strategy for cancer treatment although there were some reports on Wnt2-inhibiting therapy.[35,36]

The siRNA sequence selected in the present study targets both *Wnt2B2* and *Wnt2B1*. As a result, the Ad-shWnt2B effectively inhibited the *Wnt2B2* expression in various

Wnt2B2-overexpressing tumour cells. Furthermore, the transduction with Ad-shWnt2B downregulated c-*Myc* and *survivin* in *Wnt2B2*-overexpressing A549 cells. The downregulation of c-*Myc* and *survivin* can inhibit tumour growth and induce tumour apoptosis. Consequently, the transduction with Ad-shWnt2B inhibited the growth of *Wnt2B2*-overexpressing tumour cells both *in vitro* and *in vivo*. Although we evaluated c-*Myc* and *survivin* expressions in Hela cells and PANC cells, the transduction with Ad-shWnt2B did not strongly downregulated c-*Myc* and *survivin* in these cells. Further studies including the Frizzled receptor family and other Wnt-target genes should be performed to clarify the precise mechanism.[37–39]

The percentage of viable cells continuously decreased due to the durability of the inhibitory effect of the shRNA-expressing adenoviral vector. As a result, the adenoviral vector expressing shRNA against Wnt2B has an effective antitumour activity against *Wnt2B2*-overexpressing tumours. Further studies including non-viral vectors may be required to develop this new treatment strategy for patients with *Wnt2B2*-overexpressing tumours.[40]

Conflict of interest statement

None declared.

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