

# Molecular targeting of neuroblastoma with a novel p16<sup>INK4a</sup> transporter system

TAKUYA KAWAGUCHI<sup>1</sup>, KAZUHIRO YOSHIKAWA<sup>2</sup>, KEIJI KAWAMOTO<sup>3</sup>,  
KUNIKAZU YOSHIMURA<sup>4</sup>, HIDEYUKI OSHIGE<sup>4</sup> and AKIO ASAI<sup>4</sup>

<sup>1</sup>Department of Neurosurgery, Kansai Medical University, Moriguchi City, Osaka 570-8507;

<sup>2</sup>Second Department of Pathology, Aichi Medical University School of Medicine, Yazago, Nagakute City, Aichi 480-1195; <sup>3</sup>Higashi Oosakayamaji Hospital, Higashiosaka City, Osaka 578-0925;

<sup>4</sup>Department of Neurosurgery, Kansai Medical University, Hirakata City, Osaka 573-1191, Japan

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**Abstract.** Potential molecular targets in neuroblastoma include ALK mutations, p16 deletion and CDK2A mutations; however, targeted therapeutics have not been developed for these factors. We developed Wr-T, a new system for intracellular peptide and protein delivery with a 30-residue sequence that mediates molecule entrapment and intracellular permeability. Wr-T was used to introduce the p16<sup>INK4a</sup> functional peptide to restore the tumor suppressor function of p16<sup>INK4a</sup>. Introduction of Wr-T into rats with subcutaneous grafts of neuroblastoma produced an astonishing 75.6% tumor suppression ( $p < 0.0005$ ). Thus, the p16<sup>INK4a</sup> functional peptide can be introduced in low doses and, because it exists *in vivo*, it should produce fewer side-effects than standard chemotherapy. We suggest this system could be used for molecular-targeted peptides other than p16<sup>INK4a</sup> and should be pursued for further development.

## Introduction

Neuroblastomas (NB) are tumors that arise from the embryonic neural crest. In Japan, NBs are the most common childhood malignancy after leukemia and the most common solid malignant tumor in childhood. Differentiation and maturation of tumor cells occurs during the clinical course, and spontaneous tumor regression is sometimes observed. Prognosis differs with age at onset; while the prognosis is favorable for children up to 12 months old, the prognosis is poor for children past the age of 12 months. Three-year overall survival in the high-risk group is >30%, based on the Children's Oncology Group (COG) risk stratification (1).

Therapeutic strategies for NB have always been based on risk. For high-risk patients, multidisciplinary treatment is necessary; in addition to surgical resection, treatment includes proactive chemotherapy, high-dose chemotherapy + autologous hematopoietic cell transplantation and radiotherapy (1,2). However, even with these treatments, 3-year progression-free survival is low at 20-40% and new, effective treatments are needed (3-5).

In the 1980s, MYCN gene amplification was reported to be a cause of NB (6). MYCN gene amplification, an abnormality observed in ~10% of NB cases, has been closely correlated with NB prognosis (7). However, the development of therapeutic agents targeting this gene is not easy, and no progress is being made. ALK mutations in NB were reported in 2008 (8-11). Although research on ALK inhibitors is underway, this research has not led to the discovery of molecular-targeted drugs (12). Mutations in the CDKN2A gene in NB cell lines have led to dramatic reductions in the production of p16<sup>INK4a</sup> protein (13,14). Another report confirmed the expression of p16 protein in neuroblastoma cell lines and cited reduction in 12 of 19 cases (63%) (15). Non-function of the cell cycle shutdown system due to absence of p16<sup>INK4a</sup> at the G1 checkpoint is also considered a possible cause of NB.

Recently, protein transduction domain systems (PTDs) have been garnering attention as an effective and safe system for intracellular drug delivery. PTDs can pass through the membranes of living cells and are thus useful for introducing molecular-targeted functional proteins and peptides into cells. Many PTDs have been reported, such as HIV-1 TAT (16), pAntp43-58 (17) and polyarginine (9,18). In 1998, Nagahara *et al* created a recombinant fusion of TAT and p27<sup>kip1</sup>; the fusion protein was delivered intracellularly, where it induced G1 arrest and cell migration (16). Kondo *et al* developed a system for introducing PTDs into cells in which, rather than directly binding PTDs to functional peptides or proteins, they synthesized individual functional peptides and the peptides for introducing them into cells; these peptides were attached to each other by mixing. The transporter (Wr-T) is a fusion of a nine D-arginine-residue PTD and a tryptophan-rich cargo domain to which the functional peptide is attached. This system is more efficient than the previously reported

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*Correspondence to:* Dr Takuya Kawaguchi, Department of Neurosurgery, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570-8507, Japan  
E-mail: qjbtj093@yahoo.co.jp

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Pep-1 and has been used to introduce p16<sup>INK4a</sup>, the smallest functional peptide, for its antitumor efficacy in leukemia, lymphoma, glioma, and renal cell carcinoma (19).

We used Wr-T to introduce p16<sup>INK4a</sup> and determined its antitumor efficacy in NB. This method yielded strong tumor suppression in NB with few side-effects, and is thus a promising candidate for clinical application in cancer therapy.

## Materials and methods

**Cells.** Human neuroblastoma cell lines SK-N-SH, SK-N-RA, SK-H-BE, SMS-KAN, and SMS-KCNR (kindly provided by Tsukuba University), and human cervical cancer cell line HeLa were maintained in RPMI-1640 containing 10% inactivated fetal bovine serum (IBL, Gunma, Japan), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, at 37°C under an atmosphere of 5% CO<sub>2</sub>.

**Peptide synthesis.** Wr-T and r9-p16 MIS (minimum inhibitory sequence) peptides were synthesized at BioGate (Yamagata, Japan). These peptides were prepared in the HCl form following high-performance liquid chromatography purification for *in vitro* and *in vivo* applications. Peptide purity was >95%. The identity of all peptides was confirmed by mass spectrometry. To synthesize Wr-T, we used the amino acid sequence KETWWETWWTEWTEWSQGPGrrrrrrrr (r, D-enantiomer arginine), as described by Kondo *et al.* (19). To synthesize p16 MIS, the FLDTLVVLHR sequence, identified as the MIS of p16 by Fahraeus *et al.* (22), was defined as the functional core of the peptide. Since the MIS of p16 is insoluble, as is the entire p16 molecule (MIS hydrophobicity 69.2%), we fused r9 to these ten amino acids to make the conjugate less hydrophobic (hydrophobicity, 40%), thus facilitating transport into cells.

**Peptide transduction and measuring absorbance *in vitro*.** To prepare the peptide mixture for use *in vitro*, the Wr-T and r9-p16 MIS peptides were mixed in 10 µl distilled water at room temperature for 60 min (final concentration: Wr-T, 5 µmol/l; r9-p16 MIS, 8 µmol/l). The solution was added directly to 190 µl RPMI-1640 containing 5% fetal bovine serum.

Absorbance was measured on a microplate reader at 450 nm with reference at 570 nm. Dunnett's t-test was used for statistical analysis.

**PCR.** Total RNA was extracted from each human neuroblastoma cell line by using the RNeasy Mini kit (Qiagen Sciences, MD, USA). cDNA was synthesized using random primers and a cDNA synthesis kit (High Capacity cDNA RT kit, Applied Biosystems, Foster City, CA, USA). PCR was performed with AmpliTaq Gold (Applied Biosystems). Amplification conditions and primer sequences for p16, CDK4, CDK6 and cyclin D are listed in Table I.

**Western blotting.** Cells were lysed with SDS sample buffer and extracts were separated by SDS-PAGE using SuperSep™ Ace 12.5-15% bis-Tris gradient gels (Wako, Osaka, Japan) and Q-PAGE system 7.5-15% bis-Tris gradient gels (TEFCO, Tokyo, Japan). Separated proteins were transferred to a

PVDF-membrane (Immobilon-P, Millipore, Billerica, MA, USA), then sequentially probed with the following antibodies after blocking with 5% dried milk and 1% normal goat serum-PBS: mouse monoclonal anti-actin antibody (Clone: AC-74, Sigma, St. Louis, MO, USA), mouse monoclonal anti-human p16<sup>INK4</sup> (Clone: G175-1239, BD Biosciences Pharmingen, San Diego, CA, USA), mouse monoclonal anti-RB antibody (Clone: LM95.1, Oncogene), and rabbit polyclonal anti-phospho-Ser780RB antibody (Cell Signaling Technology, Beverly, MA, USA). Immune complexes were visualized with the ECL plus western blotting detection system (RPN2132, Amersham Pharmacia Biotech UK Ltd., UK) according to the manufacturer's instructions and signals were visualized and digitally captured using an image analyzer (LAS 1000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

**Flow cytometry.** Apoptosis assays were performed with the FITC-Annexin V staining kit (MBL Co. Ltd., Japan) according to the manufacturer's instructions followed by FACScan analysis (BD, Franklin Lakes, NJ, USA).

Cell cycle stage was evaluated by DNA uptake of EdU (5-ethynyl-2'-deoxyuridine). Staurosporin was used as a positive control using Click-iT® EdU Imaging kits (Invitrogen, USA).

**Mouse tumor models.** Four-week-old NOD/Shi-scid, IL-2Rγ<sup>null</sup> female mice were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). SMS-KAN human neuroblastoma cells (6x10<sup>7</sup>) in 100 l RPMI-1640 were subcutaneously injected into the flanks of each mouse to form a solid tumor nodule. Treatments were as follows: the Wr-T/r9-p16 MIS peptide mix (Wr-T, 50 nmol; r9-p16 MIS, 80 nmol) was injected into the heart of the mouse when the tumor had grown to a diameter of 5 mm (tumor volume, ~150 mm<sup>3</sup>). For control groups, 100 µl PBS without r9-p16 MIS peptide or Wr-T peptide was injected. Animal experiments were approved by the Aichi Medical University Subcommittee on Animal Research. All mouse procedures, euthanasia, and surgery, including neuroblastoma cell transplantations and peptide injections, were done painlessly or under anesthesia, within the strict guidelines of the Experimental Animal Facility of Aichi Medical University. Dunnett's t-test was used for statistical analysis.

## Results

**The p16<sup>INK4a</sup>-cyclin D1/CDK4-pRB pathway (RB pathway) in NB.** The RB pathway in cancer cells is the basic pathway that must be inhibited in order for oncogenic transformation of cells to occur. PCR and western blotting were used to verify p16, cyclin D, CDK4, CDK6, RB, and pRB in five NB cell lines. PCR revealed no p16 expression in SMS-KAN or SMS-KCNR cells, but expression was confirmed in SK-N-SH, SK-N-RA and SK-N-BE. Expression of CDK4, CDK6 and cyclin D was confirmed in all cell lines (Table I and Fig. 1A). p16 protein expression was not observed in any cell lines and RB phosphorylation, which indicates cell cycle revolution, was confirmed in all cell lines (Fig. 1B).

**Transduction efficiency and tumor suppression mechanism.** In order to examine the effects of the r9-p16 MIS peptide

Table I. PCR primers and amplification conditions.

Molecule	Sequence	Annealing temperature (°C)	Fragment size (bp)
p16	F: ATAGTTACGGTCCGAGGCC R: TGGTACTGCCTCTGGTGC	60	536
Cyclin D	F: AAAGACAGTTTTTGGGTAATCTTTT R: CCGGAGCATTGATAACCAG	55	126
CDK4	F: CTTCTGGACACTGAGAGGGC R: TGGGAGGGGAATGTCATTA	61	110
CDK6	F: CGGAGAACACCCTTGGTG R: GAGCCTGTCCAGAAGACAGC	59	105
Actin	F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAATGTACGCACGATTC	55	539

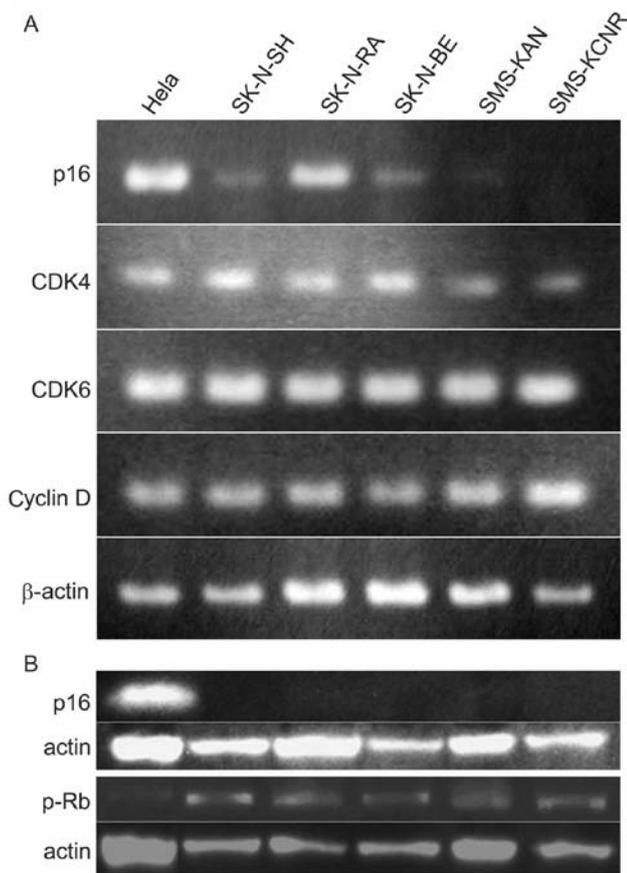


Figure 1. Expression of p16, cyclin D, CDK4 and CDK6 in NB cell lines. (A) Using HeLa cells as a positive control, we performed PCR to confirm transcript expression relative to  $\beta$ -actin. Almost no p16 expression was observed in SMS-KAN or SMS-KCNR; expression was observed in SK-N-SH, SK-N-RA, and SK-N-BE. Expression of cyclin D, CDK4 and CDK6 was confirmed in all cell lines. (B) Western blotting for phosphorylated RB as a marker of cell proliferation. Protein expression of p16 was not observed in any cell lines; RB phosphorylation was observed in all cell lines.

on the cell cycle, DNA uptake of EdU was used as a marker and Staurosporin was used as a positive control. Tumor growth suppression was verified in the SMS-KAN cell line,

which lacks p16<sup>INK4a</sup> (Fig. 4). While EdU uptake occurred in 81% of untreated cells, uptake volume in cells treated with p16 alone and Wr-T/p16 complex was markedly suppressed (40 and 30%, respectively), indicating that cell division was suppressed. Tumor growth inhibition was also verified with Annexin V-FITC and propidium iodide (PI) staining using the SMS-KAN cell line, which lacks p16<sup>INK4a</sup> expression (Fig. 5). The mechanism of cell death due was thought to be apoptosis. Twelve hours after treatment, the percentage of cells with Annexin V-positive/PI-negative staining was 2.1% in the control group, 3.6% in the Wr-T group, 5.8% in the r9-p16 MIS peptide group, and 17.9% in the Wr-T/r9-p16 MIS peptide mix group. At 24 h, the percentage of dead cells was 3.4% in the control group, 6.8% in the Wr-T group, 12% in the r9-p16 MIS peptide group, and 81.3% in the Wr-T/r9-p16 MIS peptide mix group. A mild increase was observed in the r9-p16 MIS peptide group, while the Wr-T/r9-p16 MIS peptide mix group displayed a markedly high value. These results indicate increased transduction efficiency of r9-p16 MIS peptide due to Wr-T.

We also observed the effect of r9-p16 MIS peptide on endogenous RB phosphorylation in SMS-KAN cells by western blotting (Fig. 3). A clear quantitative reduction in pRB was observed at 12 h (Fig. 3). The r9-p16 MIS peptide, which was transported efficiently into nuclei due to Wr-T, likely suppressed RB phosphorylation and arrested the cell cycle at G1 by replacing the absent p16<sup>INK4a</sup> in the RB pathway. Quantitative reduction reached its peak at 48 h after introduction, while tumor re-enlargement was observed by 96 h. While the Wr-T/r9-p16 MIS peptide mix suppressed cell proliferation through G1 arrest, the complex degrades over time, allowing RB phosphorylation to progress again.

*In vitro tumor cell suppression.* An examination of cell growth suppression in each cell line at 96 h after administration yielded no differences between the control and Wr-T groups. In the r9-p16 MIS peptide group, growth suppression rates in the SK-N-SH, SK-N-RA, SK-N-BE, SMS-KAN, and SMS-KCNR cell lines were 40.4, 28.1, 23.1, 15.1 and 33.5%, respectively. In the Wr-T/r9-p16 MIS peptide mix group, growth

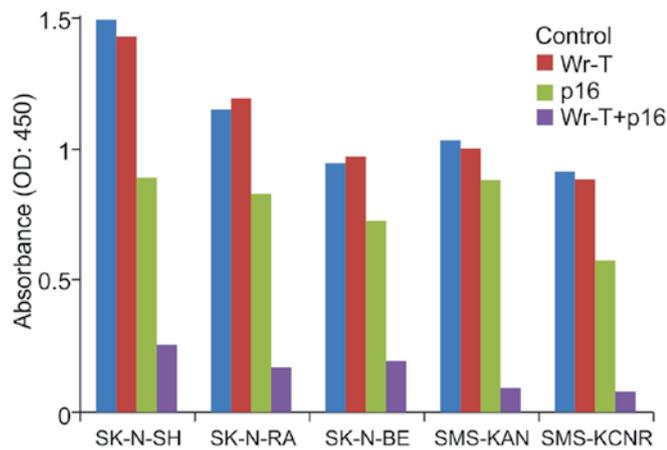


Figure 2. Growth suppression by p16. Wr-T alone, p16 peptide alone, Wr-T+p16 complex, and non-treatment control groups in all cell lines were assessed by WST-1 assays for cell proliferation after 96-h culture. There were no differences between the control group and the Wr-T group in any cell lines. In the p16 group, growth suppression rates compared to the control group in SK-N-SH, SK-N-RA, SK-N-BE, SMS-KAN and SMS-KCNR were 40.4, 28.1, 23.1, 15.1 and 33.5%, respectively. In the Wr-T+p16 complex group, a marked growth suppression was observed with growth suppression rates of 83.1, 85.4, 79.6, 91 and 91.7%, respectively.

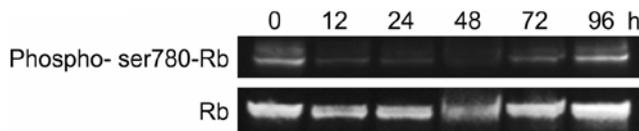


Figure 3. RB phosphorylation. We introduced the Wr-T+p16 complex into the SMS-KAN cell line and examined the cells by western blotting at various time-points. A reduction in RB phosphorylation was observed 12 h after treatment, but began to recover at 72 h.

suppression rates were 83.1 ( $p < 0.0005$ ), 85.4 ( $p < 0.0005$ ), 79.6 ( $p = 0.004$ ), 91 ( $p = 0.025$ ), and 91.7% ( $p = 0.005$ ), respectively; significant differences were observed in all cell lines. The r9-p16 MIS peptide group exhibited a marked tumor inhibition effect (Fig. 2).

**In vivo tumor suppression.** Based on the *in vitro* results, SMS-KAN cells were injected into four-week-old NOD/Shi-scid, IL-2R $\gamma^{\text{null}}$  female mice. At 14 days after tumor inoculation, tumor volumes in the control, Wr-T, r9-p16 MIS peptide, and Wr-T/r9-p16 MIS peptide mix groups were 523, 500.2, 439.3 and 127.3 mm<sup>3</sup>, respectively. Growth in the Wr-T/r9-p16 MIS peptide mix group was inhibited by 75.6% compared to the control group ( $p < 0.0005$ ) (Fig. 6A). The image shows tumors at 14 days after administration of each peptide. While similar tumors were observed in the single peptide administration groups and the non-treatment control group, only a small tumor was observed in the Wr-T/p16 complex administration group (Fig. 6B).

**Discussion**

The failure of cell cycle regulation, which functions properly in normal cells, is the cause of abnormal proliferation in cancer

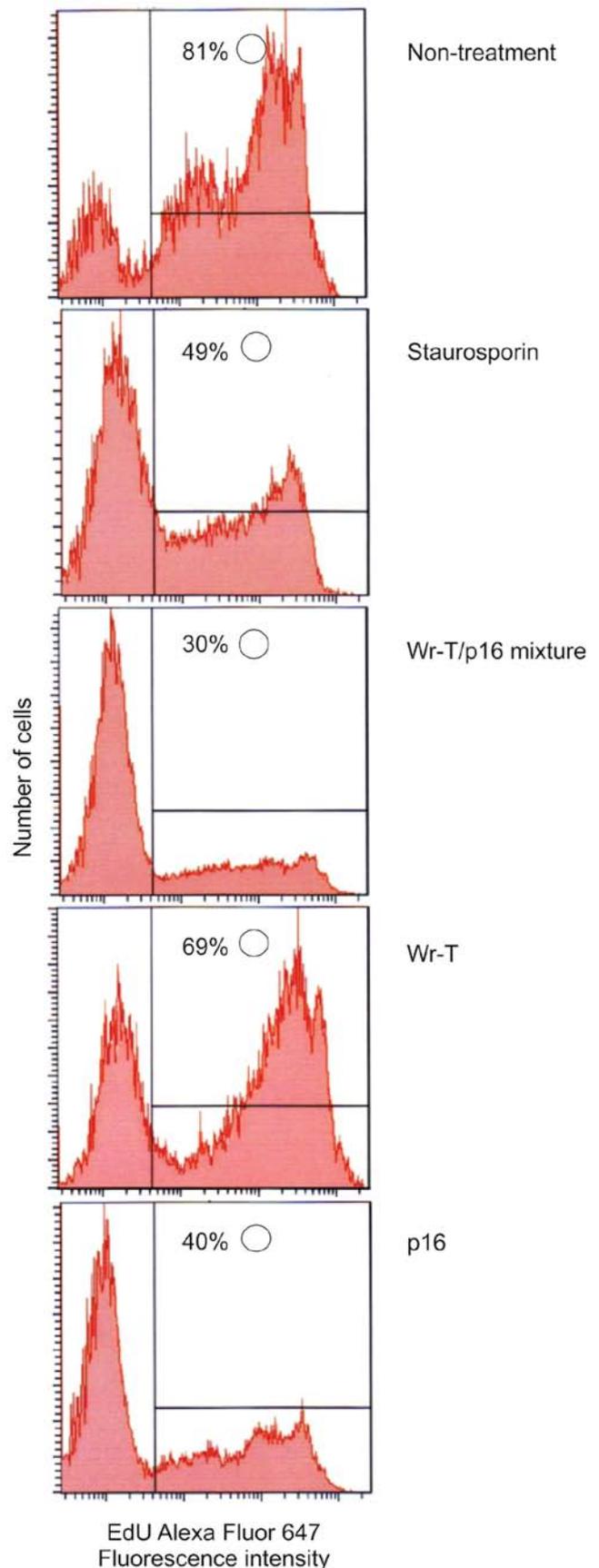


Figure 4. Cell cycle. DNA uptake of EdU (5-ethynyl-2'-deoxyuridine) as a marker of cell cycle progression in SMS-KAN cells treated with Wr-T alone, p16 peptide alone, and Wr-T+p16 complex, with Staurosporin as a positive control. EdU uptake occurred in 81% of untreated cells, and uptake volume was markedly suppressed in cells treated with p16 alone and Wr-T+p16 complex (40 and 30%, respectively), indicating that cell division was suppressed.

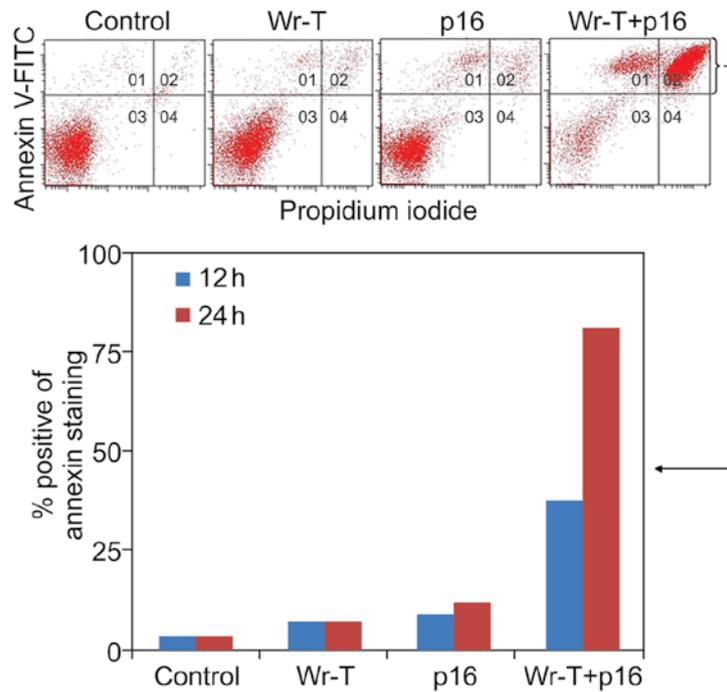


Figure 5. Annexin V-FITC and propidium iodide (PI) staining for cell death. The SNS-KAN cells were treated with Wr-T alone, p16 peptide alone and a Wr-T+p16 complex. At 12 and 24 h after treatment, we performed flow cytometry for apoptosis. In the Wr-T alone group, p16 peptide alone group, and control group, there was little increase in the count of positive-stained cells. However, in the Wr-T+p16 complex group, there was a marked increase in Annexin V-positive/PI-positive cells. At 24 h, the percentage of Annexin V/PI-positive cells had increased to 81.3%.

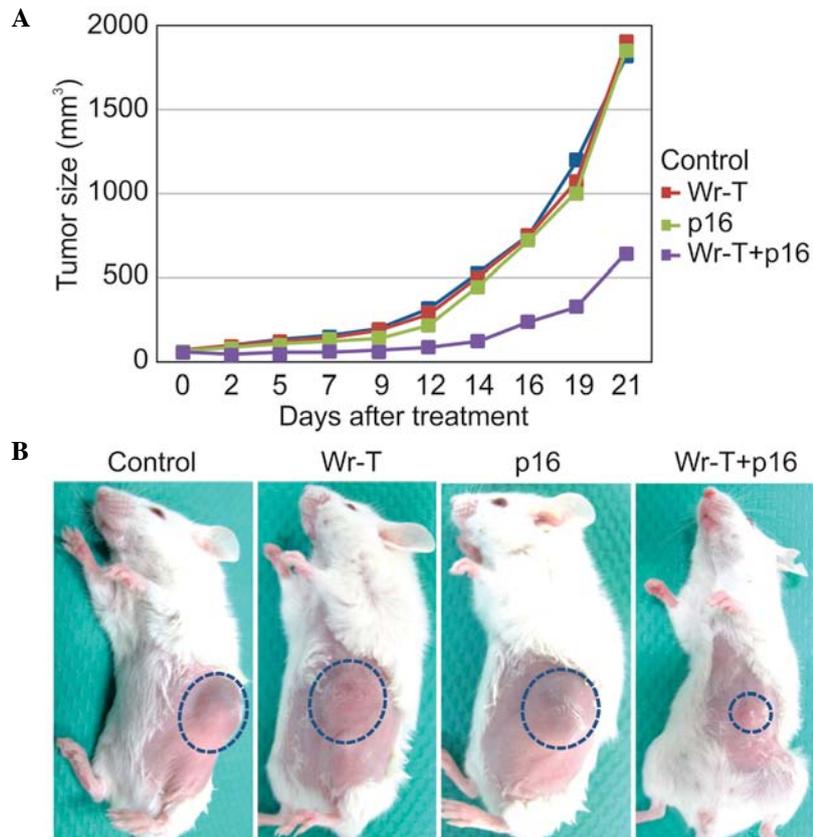


Figure 6. Tumor growth suppression by p16 in transplanted tumors. SMS-KAN cells were transplanted into four-week-old NOD/Shi-scid, IL-2R $\gamma$ <sup>null</sup> female mice. Tumor-bearing mice were treated with Wr-T alone, p16 peptide alone, or Wr-T+p16 complex and observed for changes in tumor growth. (A) The same suppression of tumor enlargement was observed in the single peptide administration groups as in the non-treatment control group. However, in the Wr-T+p16 complex group, suppression of tumor enlargement was observed; at day 14, the growth suppression effect was 75.6%. (B) Tumors at 14 days after administration of each peptide. While similar tumors were observed in the single peptide administration groups and the untreated control group, only a small tumor was observed in the Wr-T+p16 complex group.

cells. For example, cyclin-D overexpression, caused by translocation or amplification of the PRAD1 gene, promotes cell cycle revolution. The absence of cyclin inhibitors such as p16<sup>INK4a</sup> and p21<sup>cip1</sup> also promote cell cycle revolution. There are few reports on the expression of cyclin inhibitors such as p16<sup>INK4a</sup> and p21<sup>cip1</sup> in NB; however, Diccianni *et al* reported that, while alterations of p16<sup>INK4a</sup> at the genetic level are rare, they confirmed the expression of p16 protein in neuroblastoma cell lines and cited reduction in 12 of 19 cases (63%) (15).

We analyzed p16<sup>INK4a</sup> expression in five NB cell lines. Although p16<sup>INK4a</sup> expression was confirmed by PCR in three of the five cell lines, western blotting did not indicate p16<sup>INK4a</sup> protein expression (Fig. 1). Kondo *et al* reported that introduction of p16<sup>INK4a</sup> abrogated phosphorylation of retinoblastoma (RB), thereby suppressing cancer cell growth (19). We conclude that p16<sup>INK4a</sup> induces RB expression; RB phosphorylation requires normal expression of CDK4, CDK6 and cyclin D, which mediate the G1 checkpoint pathway. RB, pRB, CDK4, CDK6, and cyclin D expression was observed by PCR and western blotting in all five cell lines in which p16<sup>INK4a</sup> protein expression was absent (Fig. 1). We believe the evidence of pRB expression indicates a loss of cyclin inhibition in NB, thus producing the observed loss of growth suppression. Therefore, introduction of a cyclin inhibitor may suppress tumor growth if it inhibits RB phosphorylation. This method may have clinical therapeutic utility and differs from current therapies for NB (chemotherapy and autologous stem cell transplantation).

We synthesized the smallest functional sequence of p16<sup>INK4a</sup> and introduced it into NB cell lines using a system for transporting peptides/proteins that was previously established by our group (19-21). This system produced significant growth suppression in all five cell lines (Fig. 2). We used the same nine-arginine sequence attached to the p16<sup>INK4a</sup> peptide as described in the PTD system and observed a much greater effect with the peptide/protein transporter system complex than with the p16<sup>INK4a</sup> peptide alone. p16<sup>INK4a</sup> inhibits the function of complexes that induce RB phosphorylation (cyclin D, CDK4, and CDK6), thereby suppressing RB phosphorylation. Indeed, RB phosphorylation was suppressed in p16<sup>INK4a</sup> peptide-treated cells; we conclude the introduced peptide functioned in place of the original p16<sup>INK4a</sup> molecule. In a previous report on B-cell lymphoma, G1 arrest and apoptosis occurred in p16<sup>INK4a</sup> peptide-treated cells (19). S-phase cells are also reduced in NB, indicating that G1 arrest is occurring. While Annexin V-positive cells increase over time, no Annexin V-negative/PI-positive cells were observed in this study. We concluded that p16<sup>INK4a</sup> peptide not only inhibits cell growth but also induces tumor cell apoptosis (Figs. 4 and 5).

*In vitro* experiments have suggested the feasibility of treating NB with peptides. We tested this treatment in nude mouse models of transplanted human tumors derived from SMS-KAN cell lines. Administration of a p16<sup>INK4a</sup> peptide/transporter complex resulted in a high tumor suppression effect at 14 days (75.6% inhibition vs. controls,  $p < 0.0005$ ). However, on day 21, despite significant suppression in comparison to controls, the effect size was reduced (65% inhibition compared to the control group,  $p = 0.024$ ). Although extremely strong tumor suppression was observed after only a single administration, the tumor began to grow again;

this suggests therapeutic efficacy is limited after peptide inoculation. In a report on renal cell carcinoma, multiple administrations produced prolonged therapeutic efficacy and tumor regression. In a report on gliomas, combined administration of p14 and p16<sup>INK4a</sup> produced a strong antitumor effect (20). Based on these reports, we believe it is feasible to strive for tumor regression by optimizing combined therapies and number of administrations.

The p16<sup>INK4a</sup> functional peptide/transporter peptide complex described here was not cell-specific. Its introduction into normal and tumor cells makes toxicity a particular concern. In a report on B-cell lymphoma, peptides were introduced into normal lymphocytes, but apoptosis was not induced in these cells. In an experiment on gliomas and renal cell carcinoma in mice, no H&E staining abnormalities were observed in normal tissue surrounding the transplanted tumor or other normal organs. We also observed no abnormalities in H&E staining in the normal subcutaneous tissue surrounding the transplanted tumor or other normal tissue (brain, lungs, heart, liver, kidneys and spleen).

The introduction of a functional peptide using the transporter developed by Kondo *et al* (19) was effective in NB treatment and had almost no effect on normal cells, thus showing its therapeutic promise for further development and clinical trials.

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