

# Systemic transduction of p16<sup>INK4a</sup> antitumor peptide inhibits lung metastasis of the MBT-2 bladder tumor cell line in mice

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Received February 2, 2018; Accepted July 27, 2018

DOI: 10.3892/ol.2018.9655

**Abstract.** p16<sup>INK4a</sup> (p16) is a key molecule in bladder tumor (BT) development. We previously reported that a p16 antitumor peptide inhibited the growth of subcutaneous BT grafts in mice through restoration of p16 function using a Wr-T peptide transporter system. In the present study, the efficacy of mouse p16 peptide administration in a mouse lung metastasis model for BT and also the toxicity of peptides by cardiac peptide injection were evaluated. Mouse lung metastases were developed by tail vein injection of a p16-deficient MBT-2 cell line. Six-week-old C3H/He female mice were divided into three groups: A control group (n=12) receiving no treatment; a group treated once on the 3rd experimental day (n=12); and a group treated three times on the 3rd, 5th and 7th experimental days (n=10) with an injection of a mixture of 80 nmol mouse p16 peptide and 50 nmol Wr-T into the tail vein. At the 14th experimental day, the lung metastases were histologically evaluated. Lung metastases were observed in 100% (12/12), 41.7% (5/12) and 30% (3/10) of the aforementioned three groups, respectively. The number and area of metastatic lung tumors were significantly different between control and treatment groups (control vs. triple treatment group for the number and area, P=0.0029 and P=0.0296, respectively). Immunohistochemistry demonstrated that phosphorylated retinoblastoma (Rb) protein was decreased in lung tumors of the treatment groups, compared with the control group. The toxicity of p16 peptide

transduction was evaluated by using low-dose treatment (three dosages) and high-dose treatment (two dosages) on three male and three female C3H/He mice in early and late experimental phases. In low and high dose groups, no notable change was determined in body weight or blood analyses in early or late phases following mouse p16 peptide administration. In addition, no notable change was observed histologically in bone marrow of treatment groups. To conclude, systemic p16 peptide administration decreased lung tumor development in a mouse metastatic BT model without severe adverse events, as assessed by blood analyses and histological evaluation.

## Introduction

Human urothelial carcinoma evolves via the accumulation of numerous genetic alterations, with the loss of p53 and p16<sup>INK4a</sup> (p16) function representing important stages in the development of superficial lesions and their progression to malignant disease (1). p16 inhibits the activities of cyclin-dependent kinases, which maintain the retinoblastoma protein (pRb) in its active hypophosphorylated state (2).

A p16 gene transfection study demonstrated that growth arrest and suppression of tumorigenesis of bladder tumor (BT) cell lines may be induced by the p16 gene (3). It was reported that the p16 antitumor peptide notably inhibited the growth of highly aggressive leukemia/lymphoma types through restoration of p16 function (4). In urological cancer types, growth of renal cell carcinoma cell line graft was also inhibited by transduction of p16 peptide in mice (5). We previously reported the antitumor effect of a minimal inhibitory sequence peptide of p16 (p16-MIS) on allografts of solid tumor types, particularly urological cancer, derived from p16-deficient BT cell lines using a Wr-T system (6). In the present study, the suppression of BT metastasis with a p16-Wr-T peptide transfer system using a metastasis model of BT in mice was demonstrated, continuing on from our previous study. In terms of animal models for BT metastasis, a mouse lung metastasis model using the MBT-2 cell line was selected due to lung metastases

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**Key words:** bladder cancer, peptide transduction, lung metastasis, p16, retinoblastoma

occurring frequently, and at a similar rate to liver metastases (36 and 38%, respectively) (7). In addition, this animal model was established previously by Horinaga *et al* (8).

It has been reported that the current standard treatment for patients with systemic disease, including distant metastases of BT, is a combination of gemcitabine and cisplatin, including GC therapy (9). In addition, immune-checkpoint inhibitor, including pembrolizumab, anti-PD-1 antibody has been reported as a second treatment option for BT (10). Due to the fact that the aforementioned treatments may be associated with severe adverse events or specific immune-associated reactions, new systemic treatments are not expected to present such adverse events. Therefore, the present study also evaluated the toxicity in mice associated with systemic p16 peptide transductions.

## Materials and methods

**Cells.** The mouse BT cell line MBT-2 (Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan) was cultured in RPMI-1640 containing 10% inactivated fetal bovine serum (Immuno-Biological Laboratories, Co., Ltd., Fujioka, Japan) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The MBT-2 cell line is a p16-deficient cell line with phosphorylation of the Rb protein (6). We previously confirmed the lack of expression of p16 in MBT-2 and that restoration of p16 function by peptide transduction resulted in downregulation of phosphorylated Rb expression (6).

**Mouse model for lung metastases.** Six-week-old female C3H/He mice were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were kept under the following housing condition: 23.5±2.5°C temperature; 52.5±12.5% humidity; 200Lx illumination during daytime (5:00 to 19:00), and free access to food and water. A total of 100 µm suspension containing 1×10<sup>5</sup> MBT-2 cells in PBS was injected into the tail vein of each mouse, and lung metastases had developed when the mice were sacrificed by cervical dislocation on the 14th experimental day, based on the previous study by Horinaga *et al* (8). Horinaga *et al* (8) observed lung metastases between the 9th and 12th day after tail vein injection of MBT-2 cells and survival of mice decreased at the 15th day after injection. In addition, in our pilot study, a number of mice succumbed to severe lung metastases at the 21st day after injection (data not shown). In total, 34 mice were classified into three groups: A control group (n=12); a single treatment group (n=12); and a triple treatment group (n=10). A decreased number of mice were used in the triple treatment group due to failure (i.e., phlebitis and hematoma) of the tail vein injection during the process of experiments. The initial body weight of mice in the aforementioned three groups were 18.5±1.19, 19.2±0.970, and 18.7±0.633 g, respectively. At the end of the experiment the body weight of mice were 19.7±1.13, 20.1±0.999, and 20.0±1.22 g, respectively. Animal experiments performed in the present study were approved by the Laboratory Animal Resource Center of the University of Tsukuba (Tsukuba, Japan). All mouse procedures, euthanasia and surgery, including injections of BT cells and peptides, were conducted painlessly or under anesthesia using a combination of hydrochloric acid medetonidine 0.3 mg/kg + midazolam 4 mg/kg + butorphanol

tartrate 5 mg/kg within the strict guidelines of the Laboratory Animal Resource Center of the University of Tsukuba.

**Peptide synthesis.** All peptides including Wr-T and the r9-p16 MIS for mouse were synthesized at BioGate Co. Ltd. (Yamagata, Gifu, Japan) using standard fluorenylmethyloxycarbonyl chemistry as previously described (6). Briefly, the 10 amino acid residue sequence, FLDTLVVLHG, was identified as the MIS of p16 (11), and the amino acid sequence of the Wr-T transporter peptide was identified as KETWWETWW TEWWTEWSQGPGrrrrrrrrr (r, D-enantiomer arginine), as previously described (5,6). Peptides were purified at 95% by reverse-phase high-performance or high-pressure liquid chromatography using Shimadzu System Control HPLC 10AVP (Shimadzu Corporation, Kyoto, Japan), according to the manufacturer's protocols, and a Shimadzu C18 Analytical column (Shimadzu Corporation). Identification of all peptides was confirmed by mass spectrometry (AXIMA CFR Kratos; Shimadzu Corporation). All peptides precipitated from trifluoroacetic acid solutions are trifluoroacetic acid salts. The hydrochloride form of peptides was prepared following high-performance liquid chromatography purification.

**Peptide transduction.** *In vivo* peptide delivery to the lung metastatic tumor model of mice was performed by injecting the Wr-T/r9-p16 MIS peptide mix (Wr-T, 50 nmol; and r9-p16 MIS, 80 nmol) into the tail vein. Following the MBT-2 cell injection, the peptide mix was delivered by tail vein injection on the 3rd day or the 3rd, 5th, and 7th days for the single and triple treatment group, respectively. The control group received no tail vein injection.

**Evaluation of lung metastases following peptide transduction.** Lungs were fixed in 10% neutral buffered formalin (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan) overnight at room temperature and subsequently embedded into paraffin. Serial sections (4 µm) were used for hematoxylin and eosin (H&E) and immunohistochemical staining. The sections were stained with hematoxylin and eosin for 1.5 and 4 min, respectively, at room temperature. Lung metastases following the peptide transfer were evaluated histologically by H&E staining under a light microscope (magnification x40-400; OLYMPUS BX41; Olympus Corp., Tokyo, Japan). To evaluate lung metastases, the number and size of metastatic nodules in each group was quantified using ImageJ 1.48v (National Institutes of Health, Bethesda, MD, USA). The number of metastases was counted on random H&E-stained sections of lungs from each mouse. Additionally, the size of the tumor was represented by the area of the tumor, calculated by tracing its contour line, on the H&E-stained sections, using ImageJ 1.48 software. For the number and area of the tumor samples, the differences between groups were analyzed.

**Immunohistochemical staining of p16-associated molecules and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining transduction.** Lungs were fixed in 10% neutral buffered formalin overnight at room temperature and subsequently embedded into paraffin. According to previous studies (6,12), 4-µm serial paraffin sections were stained using a rabbit polyclonal

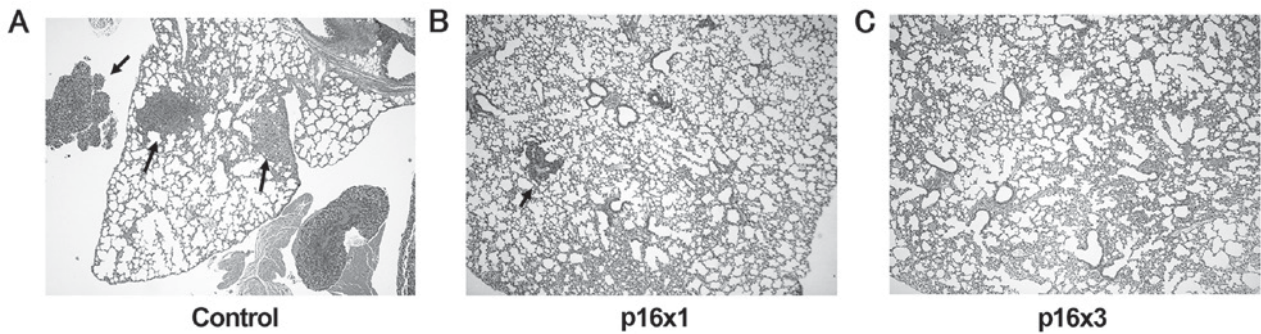


Figure 1. Histological results of the lungs of mice in (A) control group with no p16 MIS peptide treatment (B) single p16 MIS peptide treatment group (p16x1) and (C) triple p16 MIS peptide treatment group (p16x3). Histological data of the lung at the 14th experimental day after MBT-2 administration via the mouse tail vein demonstrates that lung metastases are inhibited in number and size in the groups administered with the p16MIS peptide (B and C), compared with the control group (A). In the group with triple p16MIS peptide injection (C), metastases was rarely observed in the lung (magnification, x40). Note that the arrows indicate metastatic nodules.

anti-phospho-Ser780 pRB antibody (dilution, 1:100; cat. no. 9307S; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C, followed by the Universal Immuno-enzyme Polymer method using the N-Histofine Simple Stain Mouse MAX PO reagent (cat. no. 714342; Nichirei Biosciences, Inc., Tokyo, Japan), according to the manufacturer's protocol. The sections were developed with 3,3'-diaminobenzidine tetrahydrochloride containing 0.03% hydrogen peroxide for 5 min at room temperature and counterstained with hematoxylin for 90 sec at room temperature. Apoptotic cells were detected in tumors harvested from mice 48 h after the peptide administration. Apoptosis in tumor sections was determined by the TUNEL assay method with the use of ApopTag Peroxidase In Situ Apoptosis Detection kit (cat. no. S7100; Nippon Chemi-Con Co., Tokyo, Japan), according to the manufacturer's protocol. Histological findings were observed in five fields under a light microscope (magnification, x400; OLYMPUS BX41; Olympus Corp., Tokyo, Japan).

**Evaluation of toxicity of systemic p16 MIS peptide transfer.** To evaluate the toxicity of systemic p16 MIS peptide transduction, a single low or high dose of the p16 MIS peptide was injected into the hearts of mice as follows: A single low dose peptide transfer was defined as 5 nmol Wr-T and 20 nmol p16 MIS; and a single high dose was defined as 25 nmol Wr-T and 100 nmol p16 MIS. In contrast, late phase multiple high dose peptide transduction was evaluated at 12 weeks after the initial peptide administration, followed by four weekly peptide transfers of 25 nmol Wr-T and 100 nmol p16 MIS, respectively. There was no group with multiple low dose peptide transduction in late phase. Each group including the control group contained three male and three female mice, and toxicities were evaluated by body weight and blood analyses, which included a whole blood count and biochemistry, and by histological examination, including aforementioned H&E staining of bone marrow, thymus, lung, bronchus, pericardium, stomach, intestine, liver, spleen, kidney, testis, ovary and brain of mice. The mice used for toxicity evaluation were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were kept under the same housing condition previously described.

**Statistical analyses.** Each value in blood analyses and body weight was presented as the mean  $\pm$  standard deviation. Statistical analysis was performed with JMP® 9.0.0 supported

by SAS (SAS Institute Inc., Cary, NC, USA). One way analysis of variance (ANOVA) and the post-hoc Steel-Dwass test (non-parametric test) were used to compare the number and area of lung metastases, the blood analyses and animal body weight between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Inhibition of lung metastasis of BT cells following tail vein injection of p16 MIS peptide in a mouse lung metastasis model.** Due to the practical potential of the Wr-T and p16 MIS peptide delivery system, the efficacy and toxicity of this system for the treatment of lung metastasis of MBT-2 mouse BT cells in C3H mice was investigated. At the 14th day after BT cell injection, lung metastases were observed in 100% (12/12), 41.7% (5/12) and 30% (3/10) of control, single and triple doses of p16 groups, respectively. Lung tumors were notably decreased in number and size in the p16 MIS peptide transfer groups, compared with the control group (Fig. 1).

A statistically significant decrease in tumor number and size was observed in the p16 peptide groups, compared with the control group (control vs. single or triple doses of p16 MIS peptide for the number,  $P = 0.0087$  and  $0.0029$ , and in area,  $P = 0.0183$  and  $0.0296$ , of tumors, respectively; Fig. 2).

**Change in expression of p16-associated molecules and apoptosis after p16 peptide treatment.** Fig. 3A, B and C demonstrate H&E staining, immunohistochemistry using an antibody against phosphorylated Rb, and TUNEL staining in control group, respectively. In H&E staining, no microscopic change in lung metastasis was observed between control and triple p16 MIS peptide treatment group (Fig. 3D). The expression of Rb phosphorylation was notably decreased in the triple p16 MIS peptide treatment group (Fig. 3E), compared with the control group (Fig. 3B). The TUNEL staining demonstrated that apoptosis was increased in the triple p16 MIS peptide treatment group (Fig. 3F) compared with the control group (Fig. 3B).

**Toxicity of systemic transduction of the p16 MIS peptide assessed by body weight, blood analyses and histological data.** Changes in blood count and biochemistry following a single treatment in the early phase of the 2nd experimental

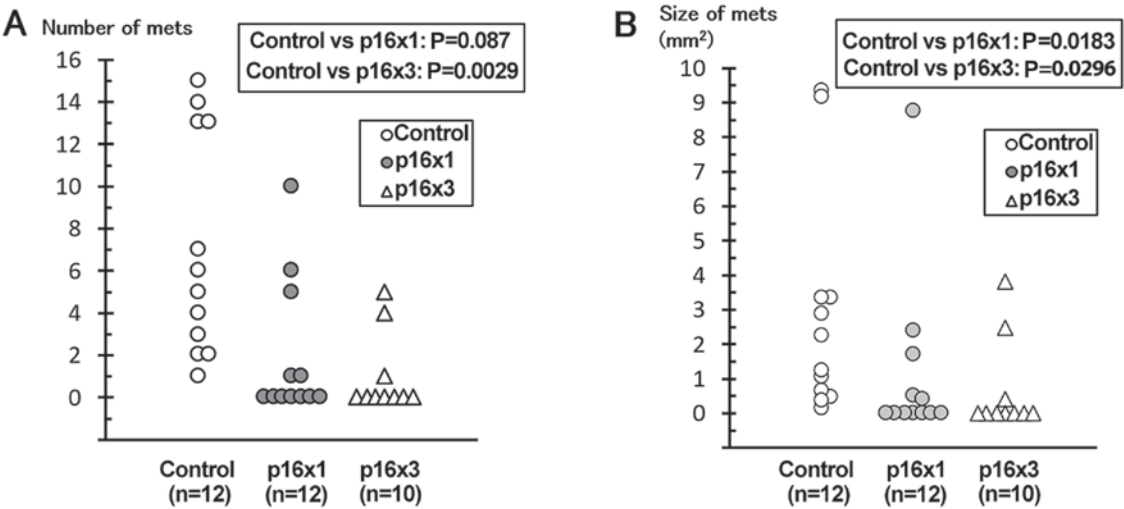


Figure 2. Number and size of lung metastases of MBT-2 cells control group and groups following p16 MIS peptide treatment. (A) Number of lung metastases in control group and groups with single and triple p16 MIS peptide treatment. For the number of lung metastases, the significance for control vs. p16x1 or p16x3 was  $P=0.0087$  and  $P=0.0029$ , respectively (B) Size of lung metastases in control group and groups with single and triple p16 MIS peptide treatment. The significance for the area of metastases was  $P=0.0183$  and  $P=0.0296$  for control vs. p16x1 or p16x3, respectively. Quantitative evaluation demonstrated that the number (A) and size (B) of metastases in histological sections were statistically different between the p16 MIS peptide transduction and control groups. p16x1, single treatment of p16 MIS peptide; p16x3, triple treatments of p16 MIS peptide.

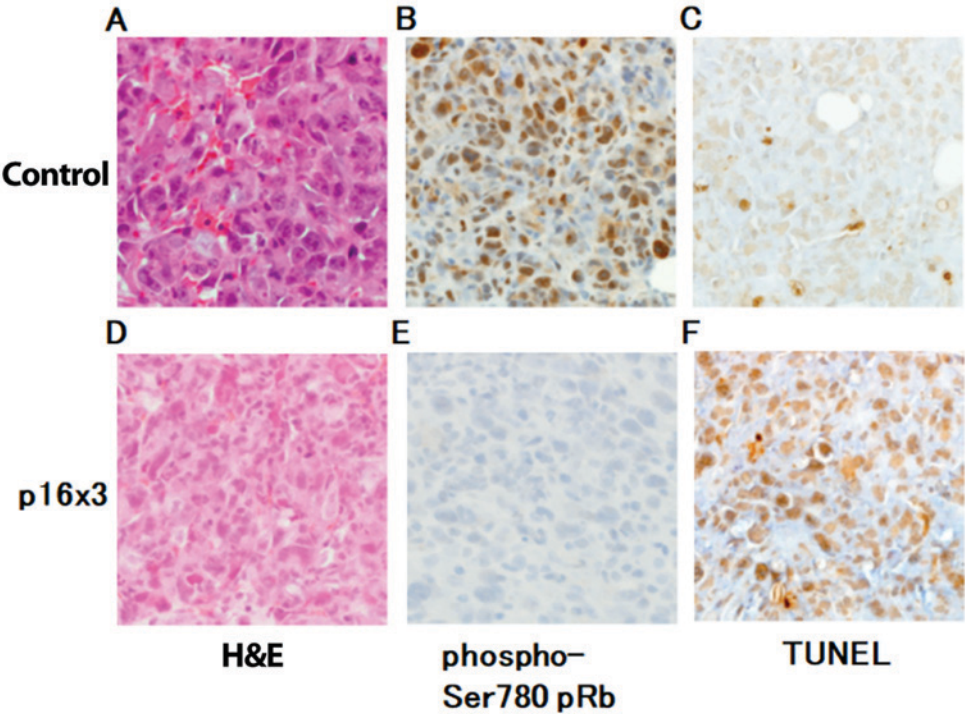


Figure 3. Histological findings of H&E staining, phosphorylated Rb immunohistochemistry and TUNEL staining in lung metastasis of control and p16 MIS peptide treatment group. (A) H&E staining of control group. (B) Phosphorylated Rb immunostaining of control group. (C) TUNEL staining of control group. (D) H&E staining of p16 MIS peptide treatment group. (E) Phosphorylated Rb immunostaining of p16 MIS peptide treatment group. (F) TUNEL staining of p16 MIS peptide treatment group. Rb phosphorylation decreased in p16 MIS peptide treatment group (E) compared with control group (B). In TUNEL staining, apoptosis was detected in p16 MIS peptide treatment group (F) compared with control group (C). Magnification, x400. H&E, hematoxylin and eosin.

week are demonstrated in Table I and indicated no significant statistical difference between control and p16 MIS peptide treatment groups in both males and females. Changes in mouse body weights during administration of multiple p16 MIS doses are displayed in Table II. No significant statistical difference was observed in body weight between groups. Additionally, Table III indicates that no statistical difference in blood tests

between the control and multiple p16 peptide treatment groups was observed in the late phase of the 12th experimental week. The white blood cell count was reduced in the group with multiple treatments, but there was no statistical difference.

There was no histological change observed in the thymus, lung, bronchus, pericardium, stomach, intestine, liver, spleen, kidney, testis, ovary, brain or bone marrow. No severe

Table I. Change in blood analyses of single treatment.

A, Male								
Group	Alb (g/dl)	ALT (U/l)	AST (U/l)	Creat (mg/dl)	WBC (/μl)	RBC (10 <sup>4</sup> /μl)	Hb (g/dl)	Plt (10 <sup>4</sup> /μl)
Control <sup>a</sup>	3.3 (0.06)	86 (23)	30 (11.5)	0.01 (0.02)	5,430 (1,036)	748 (34.8)	12.4 (0.62)	37.8 (7.0)
20 nmol <sup>a</sup>	3.5 (0.27)	74 (17)	19 (3.1)	0.02 (0.02)	5,207 (1,952)	741 (127.7)	11.7 (2.1)	35.5 (16.5)
100 nmol <sup>a</sup>	3.2 (0.15)	60 (8.7)	16 (1.7)	0.05 (0.01)	6,160 (1,384)	805 (30.6)	13.0 (0.1)	60.2 (15.0)
P-value	0.779	0.189	0.179	0.176	0.900	0.390	0.784	0.188
B, Female								
Group	Alb (g/dl)	ALT (U/l)	AST (U/l)	Creat (mg/dl)	WBC (/μl)	RBC (10 <sup>4</sup> /μl)	Hb (g/dl)	Plt (10 <sup>4</sup> /μl)
Control <sup>a</sup>	3.5 (0.28)	81 (5.0)	22 (5.6)	0.07 (0.01)	6,360 (2,786)	783 (7.07)	13.0 (0.0)	54.0 (1.78)
20 nmol <sup>a</sup>	3.7 (0.36)	91 (3.0)	19 (2.3)	0.1 (0.03)	3,430 (1,488)	873 (55.9)	14.4 (1.3)	52.2 (15.0)
100 nmol <sup>a</sup>	3.6 (0.17)	82 (1.0)	26 (5.6)	0.08 (0.02)	5,763 (1,762)	816 (27.7)	13.9 (0.06)	50.3 (8.5)
P-value	0.944	0.999	0.955	0.824	0.999	0.309	0.281	0.955

<sup>a</sup>n=3. Alb, albumin; ALT, alanine aminotransferase; AST, aspartate amino transferase; Creat, creatinine; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; plt, platelet; P16, p16<sup>INK4a</sup>; MIS, minimal inhibitory sequence; 20 nmol, the group of a single low dose peptide transfer with 5 nmol Wr-T and 20 nmol p16 MIS; 100 nmol, the group of a single high dose peptide transfer with 25 nmol Wr-T and 100 nmol p16 MIS. Each value is presented as the mean ± standard deviation (in brackets). The difference between groups was statistically analyzed by one way ANOVA and the post-hoc Steel-Dwass test (non parametric test). P-values between the control and 100 nmol groups are indicated.

myelosuppression was histologically observed in the bone marrow subsequent to systemic p16 MIS peptide transduction (Fig. 4).

## Discussion

In the present study, systemic transduction of the p16 MIS antitumor peptide successfully inhibited lung metastasis induced by venous injection in a dose-dependent manner. The decrease in the number and size of metastatic nodules, as demonstrated by histological data, indicated that the anti-metastatic effect may be based on inhibition of implantation and growth of tumor cells by cell cycle arrest (6) and that it induced apoptosis signaling. Immunohistochemistry also indicated that a candidate indicator to predict the anti-metastatic activity of the p16 MIS peptide could be the elevated expression of phosphorylated Rb in BT cells, due to p16 expression not always being absent or decreased but also it is overexpressed in a number of BT types, as previously described (6,13,14).

In previous studies regarding *in vivo* transduction of the antitumor peptide, adverse effects were not investigated (6,15); therefore, in the present study, whether or not systemic transduction of the p16 MIS peptide could cause toxicities of normal organs was analyzed using blood tests and histological data. Furthermore, systemic p16 peptide transduction did

not inhibit the function of vital organs and bone marrow at the early and late phases following peptide administration. Although a number of statistical differences were observed in serum transaminase, no association with hepatic dysfunction was apparent. In H&E-stained sections, no notable histological difference was observed between control and p16 MIS peptide transfer groups at any experimental stage. This may indicate that overexpression of p16 would not suppress non-tumorous cells in mice, regardless of the activation state of proliferation.

Although the p16 MIS peptide used in the present study would be transferred into various cells in a non-selective manner, metastatic tumor in the lung could be a good candidate for non-specific transduction, for example the peptide from the present study following injection into the systemic circulation. Higa *et al* (16) developed a novel cancer-homing peptide, which has a high affinity to the cell-penetrating peptide with p16 MIS peptide, to glioblastoma cells. They screened cell-penetrating peptides as a novel biomedical delivery system and fused it with a functional p16 MIS peptide to improve therapeutic efficacy and decrease side effects on normal tissues (17,18). This system may become an ideal therapeutic system using functional peptides to improve antitumor activity against lung metastases of the present model in the future.

It may indicate that lung metastases can be controlled by the inactivation of a p16-associated cell cycle activator,

Table II. Change in body weight.

A, Male				
Group	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)
Control <sup>a</sup>	20.5 (0.305)	38.5 (3.10)	42.6 (3.30)	44.7 (5.00)
100 nmol <sup>a</sup>	21.2 (0.173)	38.2 (0.451)	44.9 (0.721)	47.5 (2.78)
P-value	0.077	0.999	0.663	0.663
B, Female				
Group	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)
Control <sup>a</sup>	20.5 (0.737)	33.3 (2.41)	35.6 (1.77)	38.0 (1.45)
100 nmol <sup>a</sup>	20.8 (0.814)	31.3 (1.34)	34.1 (4.16)	36.9 (4.17)
P-value	0.507	0.383	0.663	0.663

<sup>a</sup>n=3. P16, p16<sup>INK4a</sup>; MIS, minimal inhibitory sequence; 100 nmol, the group of multiple high dose peptide transfer with 25 nmol Wr-T and 100 nmol p16 MIS. Each value is presented as the mean ± standard deviation (in brackets). The difference between control and 100 nmol groups at each experimental day was statistically analyzed by one way ANOVA and the post-hoc Steel-Dwass test (non parametric test).

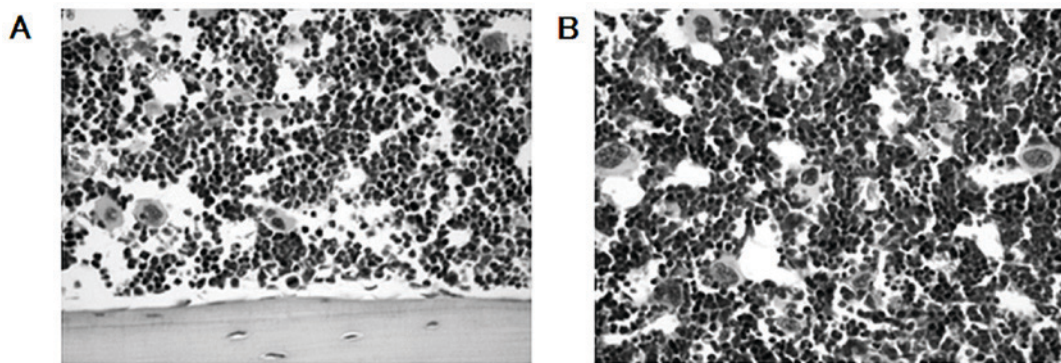


Figure 4. Histological findings of bone marrow of mice in (A) control and (B) p16 MIS peptide treatment group. No notable change was observed between control (A) and p16 MIS peptide treatment groups (B). Original magnification, x400.

including phosphorylated Rb, as demonstrated in the immunohistochemistry of the present study. Considering that p53 may be a key molecule in BT, restoration of p14 or p21, as an activator of impaired p53 function, could inhibit lung metastases. Kondo *et al* (15) previously reported that simultaneous induction of two tumor suppressor peptides (p14 and p16 or p16 and p21) suppressed the growth of a glioblastoma cell line that contained a missense mutation in p53.

Another possible strategy could be combination with a novel molecular target detected with whole genome mRNA expression profiling (19). For instance, although one of the key genes, fibroblast growth factor receptor 3 (FGFR3), has detected muscle invasive bladder cancer by the MD Anderson Cancer Center molecular subtyping (19,20), it was recently reported that FGFR3 mutations have a limited role in urothelial carcinogenesis and must collaborate with other genetic

events, including inactivation of pRb/p53 (21). Therefore, a combination therapy of functional peptide transduction with an FGFR3 inhibitor, such as BGJ398 (22), may demonstrate an enhanced inhibition of advanced bladder cancer cells in the future.

A limitation of the present study was the lack of information regarding *in vivo* kinetics in mice following administration of the p16 MIS peptide. If the accumulation of p16 MIS peptide in lung and metastatic tumor is detectable using whole-body fluorescent imaging, for example the IVIS Imaging System, it could assist with analyzing the biological modification of p16-associated molecules following peptide transduction in mice. Additionally, immunogenicity of the p16 MIS peptide should be analyzed to predict the production of antibodies against this peptide, which may be a limitation of future clinical application.

Table III. Change in blood analyses of multiple treatments.

A, Male								
Group	Alb (g/dl)	ALT (U/l)	AST (U/l)	Creat (mg/dl)	WBC (/μl)	RBC (1x10 <sup>4</sup> /μl)	Hb (g/dl)	Plt (1x10 <sup>4</sup> /μl)
Control <sup>a</sup>	3.4 (0.15)	97 (23)	23 (1.0)	0.09 (0.03)	3,843 (1,846)	882 (51.4)	13.6 (0.78)	51.9 (19.7)
100 nmol <sup>a</sup>	3.5 (0.10)	69 (19.7)	18 (2.8)	0.08 (0.03)	5,953 (1,638)	863 (30.4)	13.3 (0.43)	46.6 (13.9)
P-value	0.369	0.190	0.077	0.653	0.383	0.663	0.663	0.663
B, Female								
Group	Alb (g/dl)	ALT (U/l)	AST (U/l)	Creat (mg/dl)	WBC (/μl)	RBC (1x10 <sup>4</sup> /μl)	Hb (g/dl)	Plt (1x10 <sup>4</sup> /μl)
Control <sup>a</sup>	3.5 (0.15)	77 (18.2)	18 (4.5)	0.10 (0.02)	3,173 (867)	845 (32.2)	13.4 (0.06)	40.4 (2.2)
100 nmol <sup>a</sup>	3.4 (0.26)	72 (35.7)	20 (4.0)	0.10 (0.04)	4,230 (1,142)	861 (8.96)	13.3 (0.35)	42.7 (8.2)
P-value	0.507	0.999	0.663	0.999	0.383	0.663	0.822	0.663

<sup>a</sup>n=3. Alb, albumin; ALT, alanine aminotransferase; AST, aspartate amino transferase; Creat, creatinine; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; plt, platelet; P16, p16<sup>INK4a</sup>; MIS, minimal inhibitory sequence; 100 nmol, the group of multiple high dose peptide transfer with 25 nmol Wr-T and 100 nmol p16 MIS. Each value is presented as the mean ± standard deviation (in brackets). The difference between control and 100 nmol groups was statistically analyzed by one way ANOVA and the post-hoc Steel-Dwass test (non parametric test).

To conclude, systemic administration of the p16 MIS peptide through the tail vein significantly inhibited the number and size of lung metastases of BT in mice models. The toxicity of the p16 MIS peptide had no notable effect on the mice, according to blood and histological analyses, following short and long exposures to even a high dose of the peptide.

### Acknowledgements

The authors would like to thank Mrs Noriko Kunita and Mrs Taeko Asano, Department of Urology, University of Tsukuba for their technical support.

### Funding

The present study was supported by Grants-in-Aid from the Japan Society for the Promotion of Science, Tokyo, Japan (grant no. 24592375).

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

TS performed and analyzed the animal experiments regarding the inhibition of lung metastasis. KY analyzed animal data regarding the toxicity of the peptides. RI, TK, SK and TY

assisted with the animal experiments and its data analyses. JM analyzed histological investigation regarding the immunohistochemistry. KU supervised and analyzed molecular analysis regarding inhibition of lung metastasis. HN analyzed the molecular and histological analyses regarding inhibition of lung metastasis and revised the manuscript.

### Ethics approval and consent to participate

Animal experiments performed in the present study were approved by the Laboratory Animal Resource Center, University of Tsukuba (approval no. 12-373).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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