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A tumor-specific gene therapy strategy targeting dysregulation of the VHL/HIF pathway in renal cell carcinomas

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Hypoxia-inducible factors, key transcription factors for hypoxia-dependent gene expression, play important roles in angiogenesis and tumor growth. The VHL protein binds to the α subunit of (HIF-α) for its oxygen-dependent degradation. *VHL* mutations are found frequently in sporadic RCC. Disruption of *VHL* results in an abnormal accumulation of HIF-α, leading to the upregulation of downstream genes such as the vascular endothelial growth factor gene. We constructed a luciferase reporter vector driven by hypoxia-responsive elements (5HRE/luc) and a therapeutic vector expressing a herpes simplex virus thymidine kinase gene (5HRE/tk). In the transient transfection assay using VHL-deficient 786-O cells, constitutive luciferase expression was detected under both aerobic and hypoxic conditions. In contrast, 786-O cells transfected with a wild-type *VHL* showed hypoxia-inducible luciferase activity. In *in vitro* MTS assay, 50% of growth inhibition of 786-O cells stably transfected with 5HRE/tk was achieved with exposure to 0.2 μg/mL of GCV under both aerobic and hypoxic conditions. Xenografts of the stable clone in SCID mice exhibited a marked regression on daily injections of GCV (50 mg/kg) for 10 days. In conclusion, a hypoxia-responsive vector may have therapeutic potential for RCC with *VHL* mutations. (*Cancer Sci* 2005; 96: 288–294)

Hypoxia-inducible factors, known as transcription factors, control the expression of genes that play important roles in angiogenesis and tumor growth.[1–3] HIF are composed of a heterodimer of α and β subunits. The α subunit of HIF (HIF-α) is regulated tightly by oxygen availability, while the β subunit (HIF-1β) is expressed constitutively. Three HIF isoforms (HIF-1α, HIF-2α and HIF-3α) similar in structure and binding capability to HIF-1β have been identified.[3] HIF activate transcription by binding to the HRE, which was originally reported in the 3′ flanking region of the human and mouse *Epo* genes.[4,5] Similar HIF binding sites have been found in regulatory regions of other hypoxia-inducible genes, such as *VEGF*, *EPO* and *GLUT-1*.[6–8]

The molecular mechanisms behind the regulation of HIF have been elucidated by recent studies, and indicate that the VHL protein forms an E3 ligase complex in association with elongin B, elongin C, Cul2 and Rbx1,[9–13] which binds to HIF-α for its oxygen-dependent degradation via the ubiquitin–proteasome pathway.[14–17] Furthermore, binding of VHL to HIF requires the hydroxylation of several proline residues within the oxygen-dependent domain of HIF-α in the presence of oxygen.[18–21]

Mutations of the *VHL* tumor suppressing gene are associated with the development of multiple tumors, including hemangioblastomas in the central nervous system, RCC and pheochromocytomas.[22] In sporadic clear cell RCC, which accounts for 75% of RCC,[23] the *VHL* gene was mutated in 33–57% of cases[24–28] and silenced by hypermethylation in an additional 15–19%.[29,30] Interestingly, most of the *VHL* mutations in RCC were located at a particular site within exon 2 encoding the HIF-binding β domain.[31] Thus, disruption of *VHL* results in a marked increase in HIF-1 and/or HIF-2 activity in non-hypoxic conditions because of the impaired VHL-dependent degradation of HIF-1α or HIF-2α, leading to the upregulated expression of VEGF, GLUT-1 and EPO, as demonstrated by studies using VHL-deficient cell lines,[32,33] and clinical samples of RCC.[34–37] From these findings, we speculated that the dysregulation of HIF-α caused by *VHL* mutation in RCC might be exploited as a potential therapeutic target.

Gene therapy has been used in clinical trials for cancer treatment. One of the current problems with cancer gene therapy is the poor targeting selectivity of vectors, leading to a low efficiency of gene transfer to tumor cells and an increased risk of normal tissue toxicity. A tumor-specific gene therapy targeting aberrant transcriptional control may be a solution because the use of tumor-specific promoters can regulate the expression of therapeutic genes at a specific site or in a particular tumor. Several published studies have shown vector systems targeting hypoxic regions within solid tumors, utilizing HRE derived from mouse phosphoglycerate kinase-1,[38] mouse *VEGF*[39] and *EPO*.[40] In an earlier study, a vector construct using five copies of HRE derived from the human *VEGF* gene promoter ligated to a hCMVmp (5HRE/hCMVmp) conferred a marked increase (over 500-fold) in responsiveness to hypoxia in human fibrosarcoma HT1080 cells.[41] Based on this hypoxia-inducible promoter system, a therapeutic model targeting tumor hypoxia was established using the gene for *Escherichia coli* NTR, a prodrug-activating enzyme.[42] HT1080 cells stably transfected with the 5HRE/hCMVmp-NTR vector showed hypoxic induction of NTR gene expression in correlation with increased sensitivity to *in vitro* exposure to the prodrug, and a growth delay was observed in tumor xenografts of the same stable transfectants treated with both intraperitoneal injection of the prodrug and respiration of hypoxic gas.[42] From these findings and results, we expected the hypoxia-inducible vector to be useful for targeting dysregulation of HIF in VHL-deficient RCC as well. The purpose of this study was to test the therapeutic potential of the hypoxia-inducible vector system for RCC harboring *VHL* mutations.

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Abbreviations: CMV, cytomegalovirus; EPO, erythropoietin; GCV, ganciclovir; GLUT-1, glucose transporter 1; HA, hemagglutinin; hCMVmp, human cytomegalovirus minimal promoter; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; HSV, herpes simplex virus; HSVtk, herpes simplex virus thymidine kinase gene; luc, luciferase; NTR, nitroreductase; PBS, phosphate-buffered saline; RCC, renal cell carcinoma; RT–PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecylsulfate; tk, thymidine kinase; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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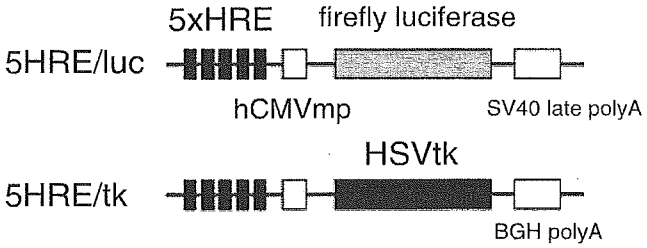

Fig. 1. Structure of hypoxia-inducible plasmids. The constructs of the hypoxia-inducible reporter plasmid 5HRE/luc and the therapeutic plasmid 5HRE/tk are indicated.

Materials and Methods

Hypoxia-inducible vectors. A hypoxia-inducible vector expressing a firefly luciferase gene with a backbone of pGL3 (Promega, Madison, WI, USA) (5HRE/hCMVmp/luc) was constructed previously.[41,43] To generate a 5HRE/tk therapeutic vector, the luciferase gene in the 5HRE/hCMVmp construct with a backbone of pEF/cyto/myc (Invitrogen, Carlsbad, CA, USA) as shown previously,[42] was replaced with a human HSVtk gene (Invivogen, San Diego, CA, USA). Each plasmid construct is shown in Fig. 1.

Cell cultures and hypoxic treatment. Human RCC 786-O cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and 786-O cells stably transfected with either HA-tagged wild-type *VHL* (786-O/VHLwt), HA-tagged truncated *VHL* 1–115 (786-O/VHLmt) or an empty vector (786-O/VHL(–/–)), were provided by Dr Kaelin WG Jr, and are presented as WT8, 115–3, and pRc3, respectively.[44] Untransfected 786-O cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, while the transfected cells were maintained in the same medium and serum containing 800 μg/mL of G418. Human fibrosarcoma HT1080 cells were maintained in MEMα containing 10% fetal bovine serum. For aerobic incubation, cells were cultured in a well-humidified incubator with 5% CO_2 at 37°C. For hypoxic treatment, cells were cultured in a Bactron II anaerobic environmental chamber (Sheldon Manufacturing, Cornelius, OR, USA) maintained with 90% N_2, 5% H_2 and 5% CO_2.

Transient transfection and luciferase assay. Cells (1×10^5) were seeded in six-well plates 24 h before transfection. Transfection was carried out with 2 μg of 5HRE/luc, 0.04 μg of the control pRL-CMV plasmid (Promega) and 6 μL of Superfect Reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions. The medium was replaced with a fresh batch 3 h after transfection. After incubation for 16–20 h, the cells were trypsinized and split. They were then incubated 10–12 h before either hypoxic or aerobic incubation for 18 h. Cell lysates were then prepared with 400 μL of passive lysis buffer using a Dual luciferase assay kit (Promega). Luciferase activity was measured using a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany).

Immunoblotting analysis. Cells (2×10^5) were seeded in a pair of six-well plates. The next day, one of the plates was kept under hypoxic conditions and the other under aerobic conditions for 18 h. Cells were then collected in 100 μL of 1 × sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and heated at 95°C for 5 min when 20 μL of each sample was immediately loaded on a SDS polyacrylamide gel (10% for VHL detection; 7.5% for HIF-2α detection) and separated by electrophoresis. To achieve an equal amount of loading between samples, protein volumes were normalized by cell number. Proteins were transferred to a nitrocellulose membrane, blocked with 5% nonfat milk in Tris-buffered saline, and incubated with 0.2 μg/mL of anti-HA antibody (Roche Diagnostics, Indianapolis, IN, USA) for VHL detection or with 0.1 μg/mL of anti-EPAS1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h for HIF-2α detection. Detection was carried out with a chemiluminescence-based method using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA).

Stable transfection. To establish stable transfectants of 786-O/VHL(–/–), 786-O/VHLmt, 786-O/VHLwt and HT1080 with the 5HRE/tk vector, 3×10^5 cells were seeded and stably transfected with both 10 μg of 5HRE/tk plasmid and 1 μg of pEF6/Myc-His-A plasmid, which expresses a blasticidin-resistance gene, by a modified calcium-phosphate method. The cells were then trypsinized 24 h after transfection and cultured in the selection medium containing 5 μg/mL blasticidin for 10 days. After selection, the mixtures of each blasticidin-resistant cell were used directly for both RT–PCR analysis and *in vitro* proliferation assay without the isolation as a clone. To establish stable clones of 786-O cells with 5HRE/tk vector, 3×10^5 of 786-O cells were plated in a 6 cm dish. The next day, the cells were transfected with 5 μg of plasmid using 15 μL of Superfect Reagent. They were then trypsinized 24 h after transfection and cultured in the selection medium containing 800 μg/mL of G418. The G418-resistant colonies were isolated and used for *in vitro* cell proliferation assays and mouse xenograft assays.

***In vitro* cell proliferation assay.** One thousand cells were seeded in each well of two 96-well plates and allowed to attach overnight. Cells were treated with medium in the absence or presence of GCV (Invivogen) at various concentrations for 24 h in either hypoxic conditions for 18 h and aerobic conditions for a subsequent 6 h, or else in aerobic conditions for 24 h. The medium was then replaced with fresh medium without the GCV, and subsequent aerobic incubation was carried out for an additional 72 h. Growth inhibition was determined by colorimetric quantification using a Celltiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega). Briefly, 10 μL of MTS tetrazonium solution (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) was added to each well. After incubation for 2 h, absorbance at 490 nm was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated as the ratio of the absorbance value at each condition against that incubated in medium without GCV under continuous aerobic conditions.

Semiquantitative RT–PCR analysis. Blasticidin-resistant stable transfectants of 786-O/VHL(–/–), 786-O/VHLmt, 786-O/VHLwt and HT1080 with 5HRE/tk vector were cultured under aerobic and hypoxic condition for 18 h, and total RNA was extracted using an RNA extraction kit (Qiagen). Complementary DNA was synthesized from 2.5 μg total RNA using an oligo dT-Adaptor Primer (Takara Biomedicals, Tokyo, Japan). Primers used for PCR were HSV/tk-forward: 5′-ATA TCG TCT ACG TAC CCG AG-3′; HSV/tk-reverse: 5′-CGC ACC GTA TTG GCA AGC AG-3′; GAPDH-forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′; and GAPDH-reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′. The PCR was carried out to amplify the HSV/tk and GAPDH genes for 25 and 20 cycles, respectively. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Mouse xenograft assay. Three million cells were suspended in 100 μL PBS and inoculated in the right flank of male 6–8-week-old C.B-17/Icr-*scid* Jcl mice (Clea Japan, Tokyo, Japan). When the tumor volume had reached approximately 200 mm^3, mice were treated daily with 50 mg/kg GCV or a comparable volume of PBS by intraperitoneal injection for 10 days. Tumors were measured using a caliper and tumor volume was calculated

according to the following equation: volume = $0.5 \times a \times b^2$ (a, larger diameter; b, the smaller diameter). The study was approved by the ethical committee of the Kyoto University Institute of Laboratory Animals.

Results

Constitutive luciferase expression of a hypoxia-inducible vector in VHL-deficient and VHL-mutated RCC cells. To test the activity of a hypoxia-inducible vector in VHL-deficient 786-O RCC cells, luciferase activity was examined following transient transfection with a 5HRE/luc vector (Fig. 1). In 786-O/VHL(–/–) cells, strong luciferase expression was detected under both aerobic and hypoxic conditions. This observation was completely different from that for HT1080 cells, as shown in a previous report.[41] We also tested 786-O cells transfected with the wild-type VHLcDNA (786-O/VHLwt), and those transfected with the truncated *VHL* 1–115 (786-O/VHLmt), which is a C-terminal truncation mutant lacking a region frequently altered in sporadic and VHL-related RCC.[44] 786-O/VHLwt showed an inducible luciferase activity in a hypoxia-dependent manner, while 786-O/VHLmt showed a marked expression under both aerobic and hypoxic conditions (Fig. 2A). In addition, we examined the expression of VHL and HIF-2α in an immunoblotting analysis. A protein with the predicted size of the VHL protein was detected in each of 786-O/VHLwt and 786-O/VHLmt. HIF-2α protein was detected under both aerobic and hypoxic conditions in 786-O/VHL(–/–) and 786-O/VHLmt, while hypoxia-dependent HIF-2α expression was detected in 786-O/VHLwt (Fig. 2B), supporting the result of the luciferase assay. Thus, the expression pattern of a hypoxia-inducible vector became constitutive via mutation of the *VHL* gene.

***In vitro* cytotoxicity of 5HRE/tk influenced by different VHL statuses.** To test the therapeutic efficacy, we constructed a plasmid expressing a HSVtk gene based on the same hypoxia-inducible system (Fig. 1). The 5HRE/tk vector was introduced into 786-O/VHL(–/–), 786-O/VHLmt, 786-O/VHLwt and HT1080 cells, and stable transfectants were treated with various concentrations of GCV for 24 h under either 18 h of hypoxic followed by 6 h of aerobic conditions, or continuous aerobic conditions. The growth inhibitory effects were determined by MTS assay 96 h after the start of treatment. In 786-O/VHL(–/–) and 786-O/VHLmt, a growth inhibition rate of 50% was achieved with exposure to less than 0.2 μg/mL GCV under both aerobic and hypoxic conditions. On the other hand, the growth inhibition in both 786-O/VHLwt and HT1080 were observed only under hypoxic conditions, while no significant growth inhibition was observed with exposure up to 10 μg/mL of GCV under aerobic conditions (Fig. 3A). HSVtk transcription levels were examined using semiquantitative RT–PCR analysis (Fig. 3B). High levels of HSVtk transcripts were detected under both aerobic and hypoxic conditions in 786-O/VHL(–/–) and 786-O/VHLmt, but only under hypoxic conditions in 786-O/VHLwt and HT1080 cells, indicating HSVtk transcription levels correlated with sensitivity to GCV. From these results, *in vitro* antitumor effects of 5HRE/tk and GCV gave rise to RCC cells with mutations in the *VHL* gene both under aerobic and hypoxic conditions as well as to hypoxic cells, while cells with wild-type *VHL* were spared under aerobic conditions.

***In vivo* antitumor effects in SCID mouse xenografts of 786-O cells transfected with 5HRE/tk.** To prepare the mouse xenograft model, selected clones of 786-O transfected with 5HRE/tk were established and screened by MTS assay. Among the transfected clones, clone 9 was used for further analysis, indicating a growth inhibition rate of 50% with exposure to less than 0.1 μg/mL GCV under both aerobic and hypoxic conditions (Fig. 4A). On the other hand, the growth of untransfected 786-O cells was not inhibited by up to 10 μg/mL of GCV regardless of hypoxic treatment

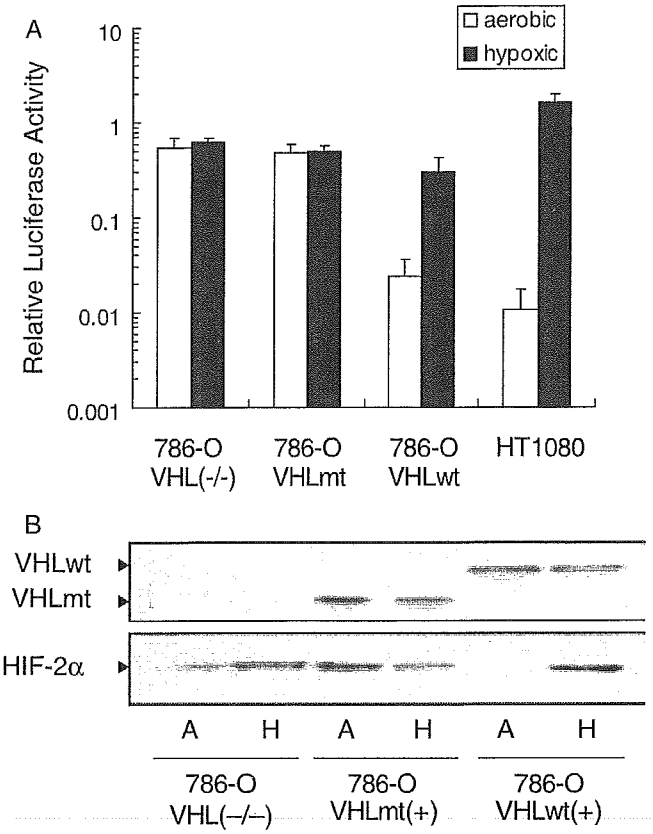

Fig. 2. Constitutive gene expression from the hypoxia-inducible promoter in VHL-deficient and VHL-mutated 786-O cells. (A) Dual luciferase assay was carried out using 786-O/VHL(–/–), 786-O/VHLmt, 786-O/VHLwt and HT1080. The cells were transiently transfected with both 5HRE/luc vector and pRL-CMV, and cultured under aerobic (open bar) or hypoxic (closed bar) conditions. To normalize the firefly luciferase activity from the 5HRE/luc vector, renilla luciferase activity from pRL-CMV was used as an internal control. The normalized luciferase activity under hypoxic conditions was divided by the one under aerobic conditions to calculate the relative luciferase activity. Results are the mean of three independent experiments ± SD. (B) Immunoblots against VHL (upper) and HIF-2α (lower) were carried out using 786-O/VHL(–/–), 786-O/VHLmt and 786-O/VHLwt cells under aerobic (A) and hypoxic (H) conditions.

(Fig. 4A). To confirm *in vivo* therapeutic efficacy, a growth delay assay was carried out. Tumor-bearing mice were treated with daily injections of GCV (50 mg/kg) or PBS for 10 days. In mice with xenografts of clone 9, marked tumor regression was observed during GCV treatment, while tumors treated with PBS continued to grow (Fig. 4B). Among the six mice treated with GCV, three mice showed tumor regrowth after cessation of GCV, while three mice showed stable tumor size (Fig. 4C). On the other hand, the tumor xenografts of untransfected 786-O cells grew regardless of GCV treatment (Fig. 4B).

Discussion

The most common type of human kidney tumors, clear cell RCC, often have mutations of the *VHL* gene, resulting in an abnormal accumulation of HIF-α and upregulation of hypoxia-dependent gene expression driven by HRE regardless of oxygen status. We have developed a hypoxia-inducible vector using HRE derived from human *VEGF* to target hypoxic cells existing in solid tumors and to overcome the resistance of hypoxic cells

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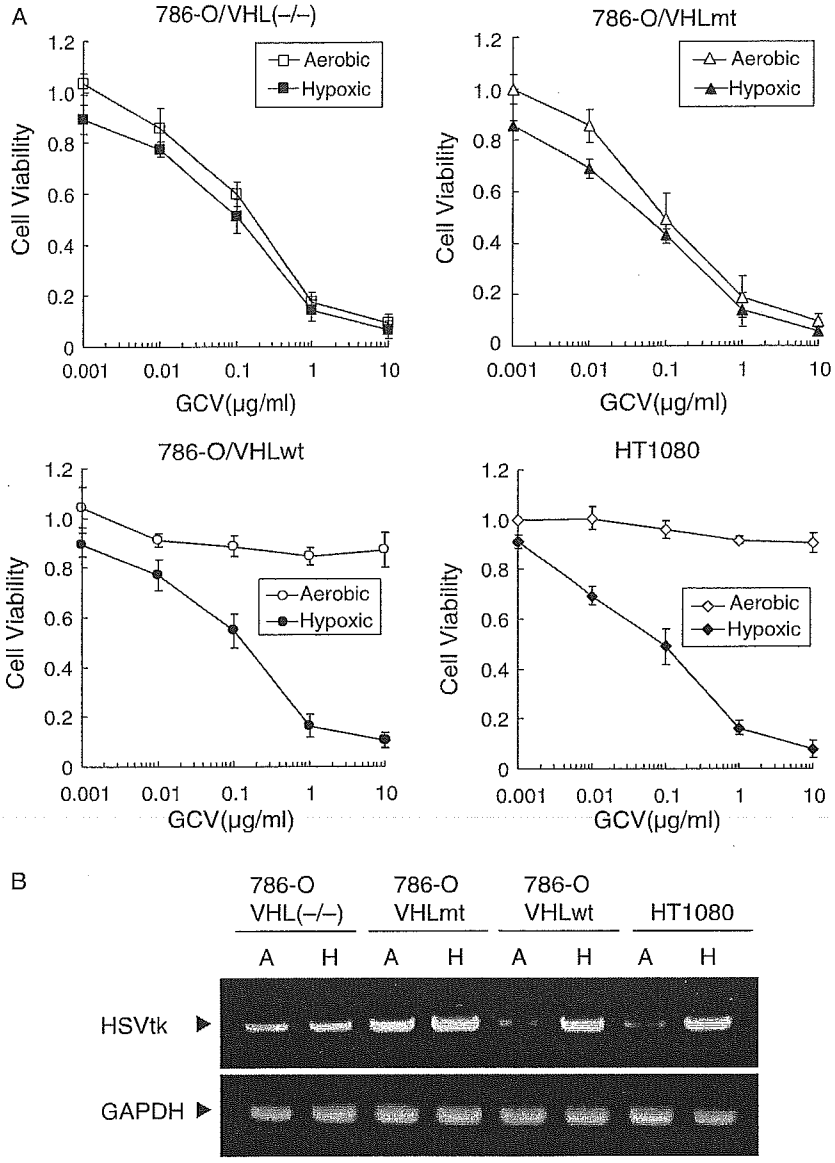

Fig. 3. Cytotoxicity of 5HRE/tk and GCV in VHL(-/-) and VHLmt cells. (A) 786-O/VHL(-/-), 786-O/VHLmt, 786-O/VHLwt and HT1080 were exposed to GCV under aerobic (open) or hypoxic (closed) conditions. Cell viability was quantified using the MTS assay at 72 h after the end of the GCV treatment, and calculated as the ratio of the absorbance value at each condition against that incubated in medium without GCV under continuous aerobic conditions. Results are the mean of three independent experiments ± SD. (B) Cells were cultured under aerobic (A) or hypoxic (H) conditions, and expression of HSVtk mRNA was assessed using semiquantitative RT–PCR using a specific primer set. GAPDH mRNA was also analyzed as an internal control.

to chemotherapy and radiotherapy. Here, we demonstrated a therapeutic model for VHL-deficient RCC using the hypoxia-inducible vector system.

First, we confirmed that HIF transcriptional activity is dysregulated by *VHL* mutations. Luciferase activity was remarkably increased in response to hypoxia in HT1080 and 786-O/VHLwt cells transfected with the 5HRE/luc vector. In 786-O/VHL(-/-) and 786-O/VHLmt, however, strong luciferase expression was detected under both aerobic and hypoxic conditions (Fig. 2A). Figure 2B shows that such dysregulation in 786-O cells is presumably mediated by constitutive HIF-2α expression, because 786-O cells lack HIF-1α.[45] These results are consistent with reports by Maxwell *et al.* and Hu *et al.*[45,46]

HSVtk is a common prodrug-activating gene used in preclinical and clinical trials. GCV is phosphorylated specifically by HSVtk to its monophosphate, which is subsequently converted to the di- and tri-phosphate forms by guanylate kinase and other cellular kinases. GCV-triphosphate can be incorporated into elongating DNA, causing inhibition of DNA replication and single strand breaks.[47] In this study, we constructed HSVtk in conjunction with the 5HRE/hCMVmp promoter as a therapeutic vector. HT1080 and 786-O/VHLwt stably transfected with 5HRE/tk showed that hypoxia-inducible transcription of HSVtk correlated with increased sensitivity to GCV, as had been demonstrated by Shibata *et al.* using the same promoter system.[42] Here, 786-O/VHL(-/-) and 786-O/VHLmt with the 5HRE/tk vector showed hypersensitivity to GCV, together with constitutive HSVtk transcription under both aerobic and hypoxic conditions. From these results, our hypoxia-inducible vector has a selective therapeutic effect not only on hypoxic cells but also on RCC with *VHL* mutations.

According to the mechanism in action described above, the HSVtk–GCV system is particularly suitable for eradication of rapidly dividing tumor cells. On the other hand, because activated GCV is an S-phase-specific cytotoxin, it is necessary that target cells must be actively dividing in S-phase at the time of exposure.[47] As shown in Fig. 4B, xenografts of 786-O cells transfected with 5HRE/tk showed a marked response to GCV and

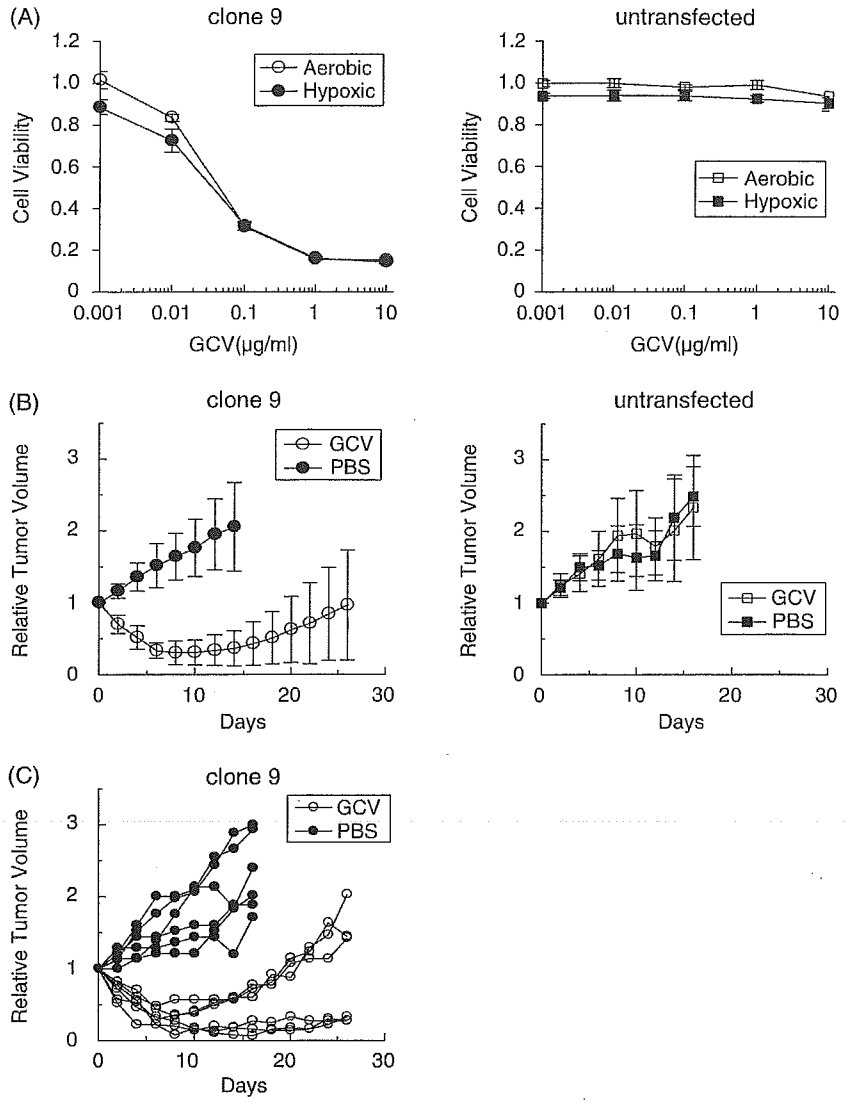

Fig. 4. *In vivo* antitumor efficacy in tumor xenografts consisting of 786-O clone stably transfected with 5HRE/tk. (A) MTS assay was carried out with the stable 786-O clone 9 transfected with 5HRE/tk (left) and untransfected 786-O cells (right), indicating hypersensitivity to GCV in clone 9 under both aerobic (open) and hypoxic (close) conditions. (B) A growth delay assay was carried out using xenografts derived from clone 9 (left) and untransfected 786-O cells (right). Tumor-bearing SCID mice were treated by daily intraperitoneal injection of either 50 mg/kg GCV (open) or a comparable volume of PBS (closed) for 10 days. Relative tumor volume as a function of days from the start of treatment is indicated. Each point and error bar is the mean ($n = 4–6$) and SD. (C) Individual tumor growth derived from clone 9 is indicated.

reduction in size during GCV treatment. However, half of them showed regrowth after cessation of GCV (Fig. 4C). This may be because elimination of activated GCV reactivated division of surviving cells that were not in S-phase during GCV treatment, and because administration dose and/or duration of GCV treatment might have been insufficient to eradicate tumors. In this experimental setting, we did not plan to use 786-O/VHLwt as a control because Iliopoulos *et al.* had already shown that 786-O subclones transfected with the wild-type *VHL* gene suppressed tumor formation in the nude mouse xenograft model.[44]

As shown in Fig. 3A, 786-O/VHLwt and HT1080 transfected with 5HRE/tk exhibited clear differences in sensitivity to GCV under aerobic and hypoxic conditions. These transfectants have no significant growth inhibition with exposure up to 10 μg/mL of GCV under aerobic conditions, suggesting the possibility of the use of the 5HRE promoter to reduce toxicity to normal tissues where hypoxic area dose not usually exist. It would be certain that the use of constitutive promoters such as CMV instead of HRE drive high expression of HSVtk in normal cells that have wild-type *VHL* even under aerobic conditions, which may damage normal cells. *In vivo* toxicity, however, remains to be evaluated with systemic administration of CMV or HRE vectors using a clinically relevant gene delivery system such as viral vectors. Binley *et al.* reported that use of the OBHRE promoter reduced hepatotoxicity with systemic administration of adenoviral vectors.[48]

In this study, we demonstrated the proof-of-principle for a therapeutic model exploiting dysregulation of the VHL/HIF pathway in RCC, providing for the potential application of a hypoxia-inducible vector system to the novel therapeutic treatment of RCC. For clinical application, however, further experiments should be conducted using gene delivery systems such as adenoviral or retroviral vectors, bacteria and macrophages. Of note, a report published during the preparation of this paper demonstrated a therapeutic effect using an oncolytic wild-type adenovirus with HRE from human VEGF gene promote on VHL-deficient RCC.[49]

In present clinical practice, patients with RCC are treated mainly with surgical resection for primary lesions. Metastatic RCC are treated with immunotherapy using interferon-α or interleukin-2, but are still difficult problems. Radiotherapy and chemotherapy are often ineffective. A tumor-specific gene therapy using the hypoxia-responsible vector system may be an option for the treatment of RCC in addition to these therapeutic

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modalities. Recently, several new therapeutic approaches for RCC have been tested in clinical trials using a radiolabeled chimeric monoclonal antibody targeting CAIX,[50] or using a neutralizing antibody to VEGF.[51]

In conclusion, the hypoxia-inducible vector system may have therapeutic potential for RCC with *VHL* mutations. Further study using delivery systems such as viral vectors, bacteria and macrophages should be conducted for clinical application.

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Review Article

Antitumor Protein Therapy; Application of the Protein Transduction Domain to the Development of a Protein Drug for Cancer Treatment

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The genomic information obtained through the human genome project has been accelerating the analysis of the functions of various disease relevant genes. The high molecular weight biomolecules, including oligonucleotides, antisense nucleotides, small interference RNA and peptides, as well as genes (cDNA) and proteins, are becoming increasingly important for the development of molecular therapies. However, the potential of such information-rich macromolecules for therapeutic use has been limited by the poor permeability across the lipid bilayer of the cellular plasma membrane. Over the past decade, a unique activity of oligopeptides, known as protein transduction domains (PTDs) or cell penetrating peptides (CPPs), has made it possible to transduce biologically active macromolecules into living cells *in vitro* by conjugating a PTD to the desired macromolecule. Furthermore, this activity has also enabled the systemic delivery of bioactive macromolecules to all tissues in living animals. However, we are now confronted with the next difficulty delivering the macromolecules specifically to the therapeutic targets *in vivo*. In this review, we focus on the application of PTD to develop antitumor macromolecules and introduce several representative strategies to discriminate between tumor and normal tissue. In addition, we discuss the unique characteristics of breast cancer, which are expected to facilitate the application of PTD to develop novel protein therapy for breast cancer.

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Key words: Protein therapy, Protein transduction domain (PTD), HIV-1 tat, Tumor hypoxia, Hypoxia-inducible factor-1 (HIF-1)

Efficient internalization of therapeutic agents into target cells is critical to gain the desired therapeutic effect. However, since the plasma membrane of the cell surface forms an effective barrier and limits the internalization of high molecular weight materials into the cells, the application of information-rich macromolecules, such as DNA and proteins, to therapies has been restricted. A variety of methods have been widely proposed to effect their delivery into living cells *in vivo* as well as *in vitro*[1-3]; unfortunately, many of them have shown inefficient delivery. In these strategies, a number of other problems, such as complex manipulation, cellular toxicity and immunogenicity, have been reported and have prevented macromolecules from routine therapeutic use.

In 1988, Green *et al.* and Frankel *et al.* separately reported that the transcriptional activator of transcription (TAT) protein from human immunodeficiency virus-1 (HIV-1) has a unique potential to enter cells in culture when added exogenously[4, 5]. The domain responsible for this translocation has been ascribed to the short basic region comprised by residues 47-57 of the TAT protein and is termed the "TAT protein transduction domain (PTD)"[6]. Subsequent studies have demon-

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Abbreviations:
PTD, Protein transduction domain; CPP, Cell penetrating peptide; HIF, Hypoxia-inducible factor; TAT, Transcriptional activator of transcription; HIV-1, Human immunodeficiency virus-1; IGF-I, Insulin-like growth factor-I; IGF-IR, IGF-I receptor; RCC, Renal cell carcinoma; VHL, Von Hippel-Lindau; CTL, Cytotoxic T lymphocyte; DC, Dendritic cell; OVA, Ovalbumin; TRP2, Tyrosinase-related protein 2; IAP, Inhibitors of apoptosis protein; Smac, Second mitochondria-derived activator of caspases; XIAP, X-linked IAP; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; ODD, Oxygen-dependent degradation; β-Gal, β-galactosidase; TOP3, TAT-ODD-Procaspase-3; AI, Apoptosis index; pO_2, Oxygen partial pressure; RNAP, RNA polymerase

strated that TAT-PTD facilitates the internalization of conjugated proteins into living cells *in vitro*[6]. Likewise, a number of the other cationic peptides, e.g. the peptides from the third α helix of the antennapedia homeodomain and from the VP22 protein of the herpes simplex virus, have been reported as PTDs showing the same attractive activity as TAT-PTD[7]. The common feature among these peptides is their highly cationic nature, which is due to their high proportion of basic amino acids, such as arginine and lysine residues[8]. Using these PTDs, various kinds of physiologically and therapeutically active macromolecules, such as peptides, proteins[9], oligo DNAs[10], super magnet beads[11], liposomes[1] and λ phages[12] have been successfully transduced into living cells. Intracellular delivery of these macromolecules modulates the functions of various genes related to the cell cycle[13] and apoptosis[14] *in vitro*. Moreover, Schwarze *et al.* demonstrated that intraperitoneal injection of a TAT-PTD-fused 120 kDa β-galactosidase (β-Gal) protein resulted in the delivery of the biologically active fusion protein to all tissues in mice, including the brain[15]. Their results opened a new possibility for the direct delivery of macromolecules into patients as protein therapy. Indeed, several groups, including ours, have applied this strategy to develop novel protein drugs to treat preclinical tumor-bearing animals[16-20].

In this review, we describe the properties and the potential of TAT-PTD as a carrier of information-rich macromolecules, and introduce representative research, in which TAT-PTD-mediated protein therapy showed significant antitumor effects with target-specificity, but without side effects. Furthermore, we discuss the possibilities of such protein therapy for breast cancer treatment.

Mechanism of TAT-PTD-Mediated Protein Transduction into Living Cells

Despite the distinctive potential of the TAT-PTD and the other arginine- and/or lysine-rich peptides as carriers of macromolecules, little is known about the mechanism involved in the cellular uptake of PTD-fused macromolecules as well as the wild type HIV-1 TAT protein.

In the early days, it was reported that no inhibition of internalization was observed at 4 ℃[21], and similar observations were reported for the basic amino acid-rich peptide derived from the antennapedia homeodomain[22]. Therefore, until recently, it was widely assumed that the PTD-mediated internalization of macromolecules occurs in an energy- and receptor-independent manner and is alternatively based on direct transport through the lipid bilayer[21]. However, it has been reported that the energy- and receptor-independence of PTD-mediated internalization are due to experimental artifacts in the process of cell fixation prior to microscopic observation and also due to the inadequate removal of proteins bound to the cell surface[23, 24]. Furthermore, it has been reported that the internalization is almost completely suppressed at 4 ℃ in unfixed conditions[24, 25]. These results, together with the observation that heparan sulfate and the inhibitor of low density lipoprotein receptor-related protein precluded the cellular uptake of PTD-fused macromolecules[25, 26], suggest that the interaction of TAT-PTD with cell surface constituents plays an important role, and is followed by an active endocytic process. Several recent papers support the involvement of an endocytic pathway in the PTD-mediated protein internalization[27, 28].

Since endocytosis is a complex mechanism including several different pathways, the identification of the critical pathway responsible for the internalization has recently been commenced. Using a permeable TAT-Cre recombinase reporter assay on living cells, Wadia *et al.* extensively analyzed the mechanism of cellular uptake of TAT-fusion protein and clearly summarized the details in 2004. After the initial ionic cell-surface interaction, TAT-fusion proteins are rapidly internalized by lipid raft-dependent macropinocytosis, but are independent of interleukin-2 receptor/raft- caveolar- and clathrin-mediated endocytosis and phagocytosis[29]. On the other hand, Richard *et al.* demonstrated in 2005 that a specific inhibitor of clathrin-dependent endocytosis partially inhibits TAT peptide uptake, implicating this pathway in TAT-peptide entry[30]. The molecular basis for the PTD-mediated cellular uptake of macromolecules into living cells still remains controversial, so further study is necessary to fully understand the process.

Development of TAT-PTD-Mediated Antitumor Protein Therapies

Research on protein transduction has dramatically expanded from *in vitro* to *in vivo* in the last decade. The advantage of this application is that we can accomplish rapid and equal distribution of PTD-linked macromolecules to all tissues and

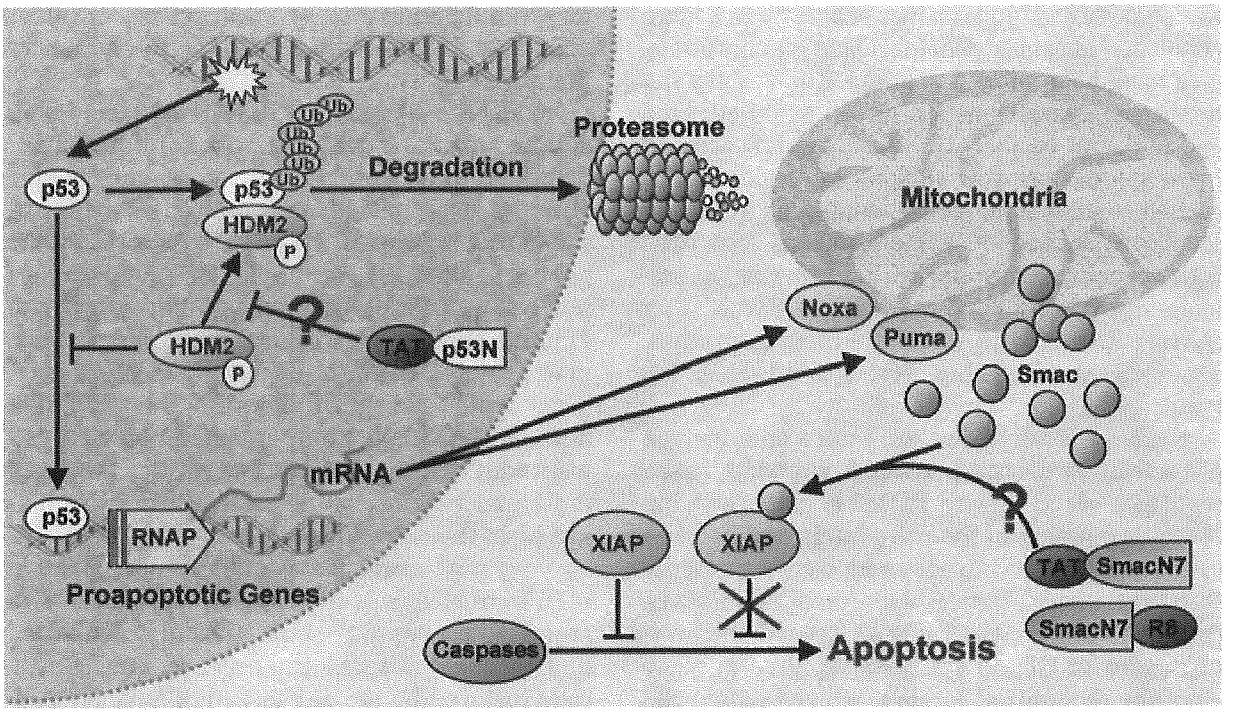

Fig 1. p53-related cellular apoptosis. HDM2 facilitates the proteolytic degradation of p53 protein. However, once damages of genomic DNA become severe, p53 is stabilized and induces various gene expressions such as Noxa and Puma and induce the release of Smac protein from mitochondria. The Smac protein interacts with XIAP and suppresses its activity, resulting in apoptosis. Since overexpression of HDM2 and IAPs inhibits apoptosis in many clinical tumors, the inhibition of their activities by p53N peptide and SmacN7 peptide respectively have been examined as novel antitumor strategies.

cells *in vivo*. However, it conversely leads to disadvantages in others. Especially for the development of PTD-fused anticancer macromolecules, the medications should have target-specificity and act locally, otherwise it may lead to damage of normal tissues and result in side effects. In the following sections, we describe representative applications showing target-specificity as well as the antitumor effect of TAT-mediated protein therapies *in vivo*.

1) Application of a Tumor Suppressor Gene, p53

Accumulated knowledge about signal cascades in cancer cells has revealed that genetic alterations of oncogenes and/or tumor suppressor genes make cells more malignant, resulting in deregulated proliferation and the evasion of apoptosis. In the development of novel cancer therapy, significant efforts at restoring the lesions that prevent the implementation of the apoptotic response have been made in order to specifically cause the death of malignant cells and in order to spare normal cells carrying few such apoptotic burdens. Such a strategy has been expected to show a much lower toxicity in normal tissue, compared with the conventional genotoxic agents that are currently in clinical use.

The gene encoding the tumor suppressor p53 is the most common anti-apoptotic lesion in cancer cells[31] and approximately 50% of human cancers bear p53 gene mutations. In most remaining cases, p53 activity is impaired by alternative molecular mechanisms, such as an elevated level of a p53 inhibitor, Mdm2[32] and the E6 protein of HPV[33], or silencing of a p53 co-activator, ARF[34,35]. One of the most important functions of p53 is "cell cycle arrest", in which p53 disturbs the replication of damaged genomic DNA and the fixation of mutations, allowing for DNA repair. Another important function is the "induction of apoptosis", which occurs in cases in which the damage to the genomic DNA is too severe to be repaired (Fig 1). These abilities of p53 are essential for the proper regulation of cell proliferation in multi-cellular organisms[35]. Loss of these functions frequently leads to cellular neoplastic transformation, and increases the resistance of cancer cells to anti-cancer therapies[36]. Therefore, restoring p53 activity in tumor cells has been expected to be an effec-

tive strategy to induce cancer cell death in a large population of cancer patients. Gene therapy strategies have been indeed conducted to restore the tumor suppressor function of p53 with both viral and non-viral vectors. However, the efficacies of these approaches were difficult to confirm under certain conditions in clinical studies as well as in preclinical studies[37, 38]. Some problems associated with immunogenicity and the low efficiency of systemic distribution were inevitable with this drug delivery system[39]. To overcome these difficulties, Tat-mediated approaches were carried out as follows.

Harbour *et al.* aimed to restore endogenous p53 activity by using a permeable peptide[18]. In the regulation of p53 activity, HDM2 interacts with the N-terminal region of the p53 protein and decreases the ability of p53 to act as a positive transcriptional factor and facilitates the proteolytic degradation of the p53 protein[40] (Fig 1). Indeed, the overexpression of HDM2 has been reported in many clinically recognized tumors, which contain the wild type p53 gene, and is associated with the functional inactivation of the p53 protein[41-43]. Therefore, it is anticipated that the disruption of the inhibitory effect of HDM2 on p53 activity would yield therapeutic benefits in tumor cells that over-express the HDM2 protein. To examine this hypothesis, the N-terminal region of the p53 protein was fused to the TAT-PTD. The resultant TAT-p53N peptide induced the rapid accumulation of p53 and the activation of apoptotic genes, and resulted in the preferential killing of tumor cells and the regression of human retinoblastoma cells in a rabbit eye[18]. Minimal retinal damage was observed after intravitreal injection[18].

2) Application of a Proapoptotic Gene, Smac

A major obstacle in cancer therapy is the resistance of cancer cells to current anticancer treatments, such as chemotherapy and radiotherapy[44]. Defects in apoptotic programs are caused by deregulated expression and function of the components of the apoptotic pathway and contribute to such resistance[45, 46]. Inhibitors of apoptosis proteins (IAPs) are frequently overexpresssed in malignant tumors[47], and they inhibit caspase activity by directly binding to activated caspase-3 and-7[48, 49]. The second mitochondria-derived activator of caspases (Smac) was identified as the protein that is released from the mitochondria to the cytosol in response to apoptotic stimuli and antagonizes IAPs to promote apoptosis[50, 51] (Fig 1). Therefore, the up-regulation of Smac activity in tumor cells may improve the resistance to anticancer therapies (Fig 1).

Fulda *et al.* examined the hypothesis using cell-permeable synthetic Smac peptides (TAT-SmacN7 in this review) containing a polypeptide from the N-terminal of Smac protein for the inactivation of X-linked IAP (XIAP)[52, 53]. As a result, the peptide enhanced the ability of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in an intracranial malignant glioma xenograft model *in vivo*[52]. Moreover, the complete eradication of established tumors and the survival of mice were achieved only upon combined treatment with the Smac peptide and Apo2L/TRAIL. In these experiments, no detectable toxicity to normal brain tissue was observed.

Yang *et al.* examined whether the inhibition of IAPs combined with chemotherapy produced synergistic effects or not[19]. First of all, they confirmed that the defect in apoptosome activity was dramatically restored by the IAP-targeting SmacN7 peptide, which is the seven N-terminal amino residues of mature Smac and has the potential to disrupt XIAP-caspase-9 interaction. On the other hand, SmacN7 peptide did not show any striking effect on the apoptosome activity of normal lung fibroblast cells. They finally demonstrated that newly synthesized SmacN7 peptide fused to the cell membrane permeable polyarginine (SmacN7R8) strongly reversed the apoptosis resistance, and displayed a synergistic effect with chemotherapy *in vivo*.

3) Application of a Tumor Suppressor Gene, VHL

Since it was reported that the growths of a variety of cancer cells are dependent on insulin-like growth factor-I (IGF-I)-mediated signaling, inhibiting the pathway has shown therapeutic effects on a variety of experimental tumor xenografts (Fig 2). For example, a truncated form of the IGF-I receptor (IGF-IR) acts as a dominant negative inhibitor of IGF-IR and abrogates ligand-dependent cellular transformation and tumorigenesis *in vitro* and *in vivo*[54]. Similarly, a specific IGF-IR antibody[55] and a specific IGF-IR antisense oligonucleotide[56] suppress the tumor growth and prolong the survival of tumor-bearing mice, respectively. Renal cell carcinoma (RCC) was reported to be dependent on the IGF-I-mediated signaling pathway for its growth. Previously, Datta *et al.* reported that IGF-I-mediated signaling is inhibited in the

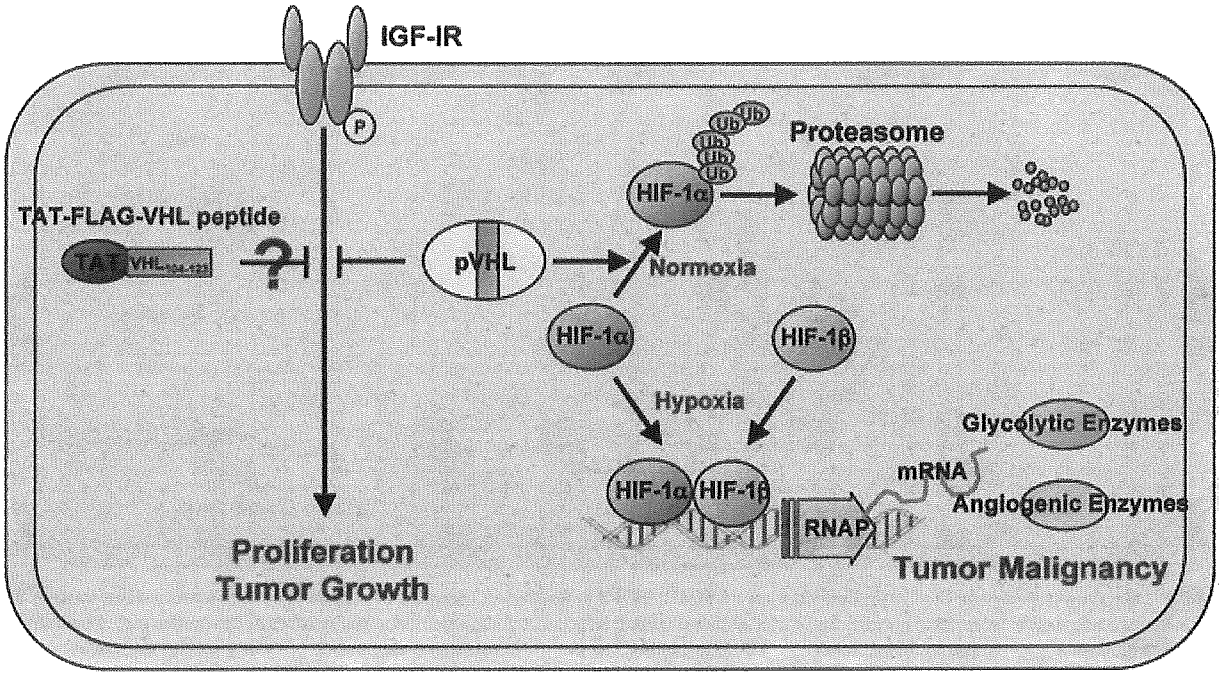

Fig 2. Function of pVHL in IGF-I-mediated signaling and in hypoxia-responsible gene expression. The growth of various cancer cells are dependent on IGF-I signaling. The signal is inhibited in the presence of pVHL in RCC cells, and thus inhibition of this signal pathway has been aimed by $VHL_{104\text{-}123}$ peptide. pVHL also acts on hypoxia-dependent gene expression. In normoxic conditions, hydroxylated HIF-1α protein is recognized by pVHL and ubiquitinated, resulting in the rapid proteolytic degradation. In hypoxic conditions, stabilized HIF-1α interacts with constitutively expressed HIF-1β and induces various gene expressions related to tumor malignancy.

presence of wild type von Hippel-Lindau tumor suppressor gene product (pVHL) in RCC cells and a specific amino acid sequence (104-123) in the β domain of the pVHL ($VHL_{104\text{-}123}$) is responsible for this function[57] (Fig 2). These results indicate that the pVHL function via the 104-123 amino acid region leads to the restricted IGF-IR signaling, resulting in restricted cell proliferation and restricted RCC growth. This is consistent with the reports that the VHL tumor suppressor gene is mutated in the majority of patients with RCCs, as well as in patients with VHL disease[58, 59], and that the mutations are located in one of the hotspots of the VHL gene[60]. In addition to a well-known function of pVHL to regulate the stability of hypoxia-inducible factor-1α (HIF-1α) protein[61], this activity must also play an important role in the tumorigenesis of RCCs.

Based on this preclinical research, Datta *et al.* examined the effects of $VHL_{104\text{-}123}$ on tumor characteristics[20] (Fig 2). $VHL_{104\text{-}123}$ conjugated to the TAT-PTD (TAT-FLAG-VHL peptide) inhibited the thymidine incorporation into RCC cells by nearly 80% compared with a counterpart protein (TAT-FLAG). Furthermore, the TAT-FLAG-VHL peptide inhibited the tyrosine phosphorylation of MAP kinase, an essential downstream molecule that leads to cell proliferation. Thus, these results suggest that TAT-FLAG-VHL peptide blocks the IGF-I-induced RCC proliferation *in vitro*. Furthermore, i.p. injection of TAT-FLAG-VHL peptide retarded the growth of subcutaneous RCC tumors, and in some cases, regressed the tumors volume, and dramatically inhibited the invasiveness deeper into the muscle layer.

4) Application of a HIF-1α ODD Domain; Development of Hypoxia-Targeting Protein Drug

The genetic alterations in tumor cells directly cause the deregulated proliferation and the high metabolic demands of tumor cells, which in turn lead to the development of hypoxia in solid tumors[62, 63]. Tumor hypoxia has been recognized as a tumor specific microenvironment, in other words, healthy adults probably have few such tissues. In such conditions, a transcriptional factor, hypoxia-inducible factor-1 (HIF-1), induces various genes related to angiogenesis[64] and glycolysis[65], and leads to invasive and metastatic properties in

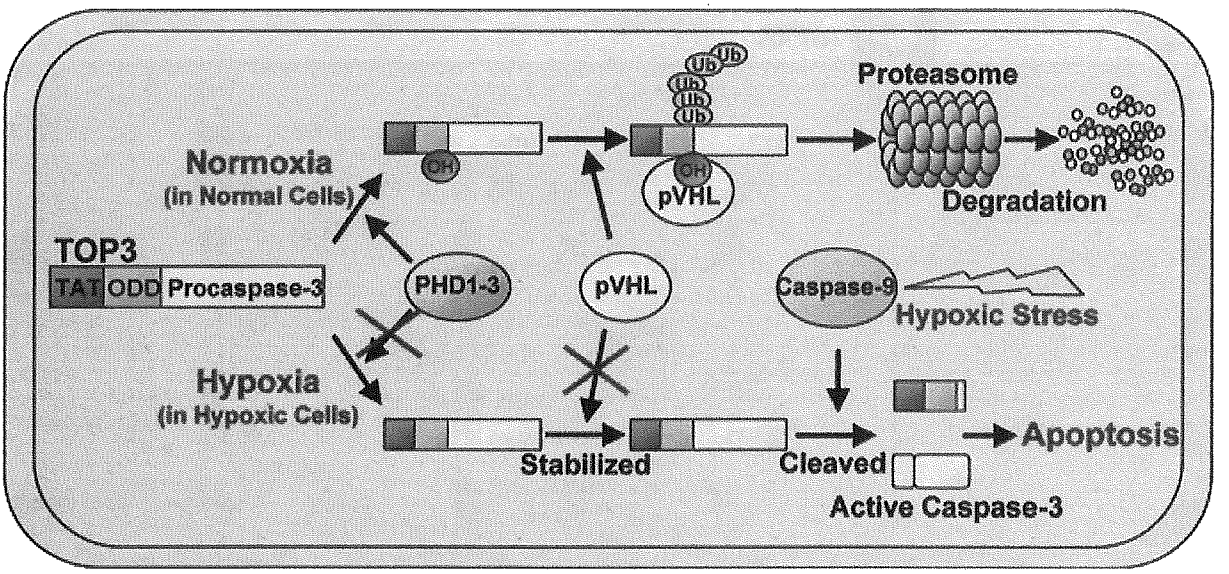

Fig 3. Hypoxia-dependent Proapoptotic Function of TOP3. TOP3 is degraded through the same ubiquitin-proteasome system as HIF-1α protein under normoxic conditions, but stabilized under hypoxic conditions. Because upstream caspases, *e.g.* caspases-9, are activated to some extent by hypoxic stress, TOP3 is cleaved to generate an active caspase-3, resulting in the enhancement of apoptotic cell death.

tumor cells[66]. HIF-1 activity is associated with the resistance of tumor cells to conventional radiotherapy and chemotherapy[67,68] and with the patient mortality in clinical studies[69-71]. Therefore, extensive efforts have been directed toward the development of novel therapies, which specifically damage the hypoxic/HIF-1-activating tumor cells[68,72].

HIF-1 is a heterodimeric transcriptional factor composed of an alpha subunit (HIF-1α) and a constitutively expressed beta subunit (HIF-1β)[73] (Fig 2). HIF-1α expression is tightly regulated at the post-translational level by oxygen-dependent prolyl hydroxylation and subsequent ubiquitination of its oxygen-dependent degradation (ODD) domain within the HIF-1α protein[61]. The pVHL is responsible for the ubiquitination. The stability of the HIF-1α protein is mainly responsible for the regulation of HIF-1 transcriptional activity[73].

We applied this unique property of the ODD domain to develop a novel hypoxia-targeting protein drug[16]. First of all, we identified the minimum region of the ODD domain responsible for the oxygen-dependent degradation of arbitrary proteins fused to it. As a result, we confirmed the hypoxia-dependent β-Gal and luciferase activity of ODD-β-Gal fusion protein[16] and ODD-Luciferase fusion protein (Harada *et al.*, in preparation), respectively, in the culture cell[16]. To apply the ODD-fusion protein to an *in vivo* study, we fused TAT-PTD to the N-terminal of the ODD-β-Gal protein and created a TAT-ODD-β-Gal triple fusion protein. After i.p. injection with the TAT-ODD-β-Gal fusion protein to subcutaneous tumor-bearing mice, the β-Gal activity and the existence of the fusion protein were detected only in the hypoxic regions of the solid tumor. On the other hand, they were not observed in the normal tissue. These results demonstrate that biologically active proteins can be exogenously delivered to hypoxic tumor cells by the TAT-ODD peptide *in vivo*. This was the first example demonstrating the target-specificity of TAT-mediated protein delivery. To examine whether the TAT-ODD fusion protein with cytotoxicity shows antitumor effects or not, the TAT-ODD peptide was further fused to a pro-apoptotic protein (Fig 3). We intentionally chose a precursor of caspase-3, procaspase-3, because it is activated in response to hypoxic stress, which was thought to reduce the possibility of side effects in the well-oxygenated normal tissues (Fig 3). Systemic administration with the resultant fusion protein, TAT-ODD-Procaspase-3 (TOP3), reduced the tumor mass as well as suppressed the tumor growth without any obvious side effects in tumor-bearing mice. The hypoxia-targeting effect of TOP3 was proven using a rat ascites model, in which intraperitoneal injection with MM1 cells results in highly hypoxic ascetic fluid[17]. Inoue *et*

al. demonstrated that intraperitoneal injection with TOP3 resulted in a significant increase in the lifespan of rats with the malignant ascites, and furthermore, 60% of the treated animals were cured without the recurrence of ascites.

5) Other Possibilities for the Development of TAT-PTD-Mediated Antitumor Protein Therapy

Several *in vitro* studies have reported other possibilities which may enable delivery of the biologically active macromolecule specifically to the desired tumor *in vivo*[74,75]. First, the fact that PTDs selectively interact with distinct glycosaminoglycan species may allow targeting of selective tissues that differ in their surface-expressed glycosaminoglycan patterns[27]. Second, by inserting a tissue- and organelle-specific cleavage recognition site between PTD and the macromolecule, PTD may be cleaved off, resulting in the accumulation of the PTD-free macromolecules in the desired tissue and organelle, respectively[76]. Third, it is also possible to generate a PTD-linked protein drug that specifically acts in tumor cells while not affecting normal cells, by applying transformed cell-specific protein activity[77]. Finally, by using a peptide that can be recognized by the tumor-specific membrane proteins, it may be possible to design a variety of proteins that specifically internalize into desired tissues.

Application of TAT-Mediated Protein Drugs to Breast Cancer Treatments

Low molecular weight chemical compounds easily pass through the cellular plasma membrane *in vitro*, and furthermore show efficient distribution *in vivo*, and thus they have been focused on for a long time in the development of anticancer drugs. Almost all of the conventional chemotherapeutic agents, however, show low target-specificity and largely affect normal tissues as well as tumors. TAT-PTD has also been reported to transduce various macromolecules to all tissues *in vivo*[15], so this technique may cause side effects, but for additional devices. Over the past decade, several modifications have been examined to achieve a tumor-specific antitumor effect of PTD-mediated strategies, as mentioned above. To apply TAT-mediated protein therapy to breast cancer treatments, it is necessary to understand the common features among breast cancers and utilize them to construct a protein drug targeting breast cancer. In the remainder of the present review, we will discuss the characteristics of breast cancer and the possibilities of PTD-mediated protein therapy targeting then.

Pusztai *et al.* identified a number of novel and routine prognostic markers of breast cancer by analyzing the gene expression profiles obtained from fine-needle aspirations of breast cancer[78]. From their list, we may be able to find a novel protein that is specifically stabilized in breast cancer, but degraded in normal tissues, and to apply such regulation to develop a breast cancer-targeting protein drug.

The role of apoptosis in oncogenesis is currently being studied intensively in breast cancer[79]. A decrease in the apoptosis index (AI) due to the overexpression of IAPs and the mutation of the p53 gene must lead to the resistance of cancer cells to current anticancer treatments, such as chemotherapy and radiotherapy[44]. Several studies have analyzed the prognostic significance of AI in breast carcinomas. Lipponen *et al.* showed a significant difference in survival from breast carcinoma ($n = 288$) depending on the AI value (cut-off point for AI was $10/mm^2$)[80,81]. Zhang *et al.* also reported a 30% difference in survival at 5 years ($p < 0.001$) in 126 patients with breast carcinoma (cut-off point for AI was $11/mm^2$)[82]. These reports indicate that the induction of apoptosis prolongs the survival of patients with breast carcinoma. In this sense, a protein, which has the potential to induce cellular apoptosis, may be a reliable candidate to be introduced into the breast carcinoma.

Chromosome 3P allele loss is a frequent event in a variety of common sporadic cancers, and breast carcinoma is no exception. To analyze the extent and frequency of 3p allelic losses in early stage invasive sporadic breast carcinoma, loss of heterozygosity analysis was carried out using a 3p microsatellite marker by Martinez *et al.*[83]. They reported that 6 out of 22 tumors showed loss at 3p25-24, including the von hippel landau locus. In such breast carcinomas, IGF-IR signaling must not be suppressed because of the VHL-deficiency, resulting in unrestricted cell proliferation. Therefore, the introduction of $VHL_{104\text{-}123}$ with TAT-PTD is expected to show an antitumor effect. Moreover, VHL-deficiency must lead to the stabilization of HIF-1α protein in such breast carcinomas. Since the stability of TOP3 is regulated by pVHL via the same ODD-regulation as the HIF-1α protein, TOP3 must be stabilized even in the aerobic regions of such breast cancers, as well as in the hypoxic regions. Therefore, we can expect addi-

tional efficacy of TOP3 toward this type of breast cancer.

The accessibility of breast carcinomas permits the use of a polarographic needle electrode to measure the oxygen tension directly in cancer patients. Such studies have shown a significantly lower median oxygen partial pressure (pO_2) in malignant tumors compared with benign tumors and normal breast tissue. The median pO_2 values in malignant tumors, in benign tumors and in normal tissues were in the range of 23-28 mmHg, 42 mmHg and 54-65 mmHg, respectively[84, 85]. Of all readings in breast cancers, 30-40% fell below 10 mmHg, which is uncommon in normal tissue. Forty % of breast carcinomas contain almost anoxic regions in the range 0-2.5 mmHg, in which tumor cells still survive[84]. In such a microenvironment, the expression of the HIF-1α protein is usually induced. Bos *et al.* reported that HIF-1α proteins were indeed accumulated in breast cancers, and furthermore, the frequency of HIF-1α-positive cells increased in parallel with the increasing pathological stage of each sample[86]. Therefore, the novel hypoxia-targeting protein drug, TOP3, will likely show antitumor effects on malignant breast cancer.

Distribution of chemotherapeutic agents from tumor blood vessels to hypoxic tumor cells is also limited and thus only poor efficacy is usually obtained in conventional cancer chemotherapy. On the other hand, biologically active proteins could be delivered into whole tumor including hypoxic tumor cells, after i.p. injection with the fusion protein genetically conjugated with TAT-PTD[16]. This result demonstrates that TAT-PTD solves the problem of the poor delivery of anticancer agents to hypoxic tumor cells. Therefore, we can expect further effects of TOP3 on hypoxic cells in breast cancer.

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9 革新的診断・治療へのアプローチ

―膜透過性・標的特異性を有する融合タンパク質を用いたイメージング・ターゲティング―

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1989年大阪大学で医学博士修得。90年学振特別研究員（がん）、93年ＥＲＡＴＯ岡山細胞変換プロジェクト研究員、99年京都大学医学研究科助手、2004年京都大学医学研究科ＣＯＥプロジェクト"病態解明を目指す基礎医学研究拠点"特任助教授。
研究テーマは、低酸素がん細胞・アポトーシスの解析と病態イメージング。趣味は旅行。

Key words：低酸素がん細胞、膜透過ドメイン（ＰＴＤ；Protein transduction domain），HIF-1，酸素濃度依存的分解（ODD; Oxygen-dependent degradation）

Abstract

低酸素が問題になる疾患には、脳・心筋梗塞や閉塞性末梢動脈硬化症といった虚血性疾患のみならず、難治性固形腫瘍が含まれる。固形腫瘍には、細胞増殖と血管新生の不均衡に起因する『低酸素領域』が存在する。低酸素領域にあるがん細胞は、放射線や抗がん剤に感受性が低く、治療効果不良の主因であるばかりでなく、浸潤・転移・再発の温床となっている。しかし、低酸素環境は正常組織には存在しないため治療標的となりうる。我々は、低酸素環境下にある細胞内で特異的に安定化する融合タンパク質を開発し、それを用いることにより、低酸素細胞のイメージング・ターゲティング研究を行っている。本稿では、固形腫瘍を対象にした研究について紹介する。

はじめに

医学の進歩に伴い、がんの診断・治療技術も年々進歩してきている一方で、我が国では、世界のどの国も経験したことのない速度で人口の高齢化が進行している。これが我が国でがんが増える第一の要因になっている。血液検査、内視鏡、画像診断等の技術の向上により、多くのがんが早期に発見できるようになってきたが、「手軽に、どのがんでも」というところまでは至っていない。我々の研究は、早期診断・早期治療に革新的イメージング・ターゲティング技術を提供することにより、がん撲滅に寄与することを目的としている。

1. 腫瘍内低酸素領域

固形がんには、非常に小さながん（数ミリ以下）でも通常ではありえないような低酸素状態のがん細胞が存在する。それは、がん細胞の増殖に血管新生が追いつかないために、血流からの酸素や栄養が十分行き渡らない領域が生じるためで、酸素も栄養も枯渇して死んでしまったがん細胞と増殖しているがん細胞の境界の極めて限られた領域に存在する（図１）。この低酸素がん細胞は、過酷な環境に順応するために、増殖は停止し、代謝も解糖系を用いて省エネルギー化し、いわば冬眠状態にある。それだけであれば、これら低酸素がん細胞は注目に値しないのであるが、これらの細胞はがん治療の抵抗性の指標となるほど、抗がん剤や放射線治療に抵抗性を示す。従って、癌治療が終わった後も生き残り、再発の温床となる可能性が指摘されている。更

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