Acknowledgments

We thank Hitoshi Kawamura and Daiju Ichimaru for helpful discussion. We also thank Yoshiko Shirakiya, Nobue Mukai, and Tomoko Sueishi for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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Telomerase-specific virotherapy in an animal model of human head and neck cancer

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Abstract

Telomerase-specific replication-competent adenovirus, Telomelysin (OBP-301), has a human telomerase reverse transcriptase promoter that regulates viral replication and efficiently kills human cancer cells. The objectives of this study are to examine the effects of OBP-301 in squamous cell carcinoma of the head and neck cells *in vitro* and in xenografted animals *in vivo*. OBP-301 was found to be cytotoxic to the YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, HN12, and KCCOR891 cell lines *in vitro*. However, the level of cytotoxicity is not correlated with the expression levels of adenoviral receptors, which may be required for adenoviral infection in squamous cell carcinoma of the head and neck cells. OBP-301 shows remarkable antitumor activity against established s.c. KCCT873 tumors in immunodeficient animals in a dose-dependent manner. In addition, no significant toxicity was observed in animals receiving treatment. These results suggest that OBP-301 is a novel therapeutic agent with promise for the treatment of human head and neck cancers. [Mol Cancer Ther 2009;8(1):171–7]

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) accounts for 5% of newly diagnosed adult cancers in the United States and 8% of cancers worldwide (1). Most patients are treated with various combinations of surgery, radiotherapy, and systemic agents (2). Despite major advances in the treatment of locoregionally advanced SCCHN, such as the introduction of novel chemotherapy regimens and inhibitors of the epidermal growth factor receptor, treatment fails in about half of the patients (3). The median survival of patients with recurrent or metastatic SCCHN who undergo chemotherapy is 6 to 9 months (4). Therefore, a considerable number of patients with SCCHN need additional treatment as the disease progresses.

Virotherapy, the approach to treat cancer with virus, has been done in some clinical trials; for example, clinical trials primarily using *p53* gene replacement (INGN-201; a replication-competent adenoviral-based vector expressing wild-type *p53*) have provided the basis for the design of ongoing randomized gene therapy clinical trials in SCCHN patients in the United States (5). Although systemic administration is probably required in the case of micrometastatic disease, virotherapy has some promise when tumor is limited to the head and neck. SCCHN is a particularly attractive model because most primary and recurrent lesions are easily acceptable to direct injection (6). Potential usage of virotherapy may include the perioperative application in the surgical wound and the addition of intratumoral (i.t.) virotherapy to current standard options, such as radiotherapy and/or chemotherapy.

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends (7). Many studies have shown the expression of telomerase activity in >85% of human cancers (8) but only in a few normal somatic cell types (9). Telomerase activation is considered to be a critical step in carcinogenesis, and its activity is closely correlated with human telomerase reverse transcriptase (hTERT) expression (10). Therefore, the hTERT proximal promoter can be used as a molecular switch for selective expression of target genes in tumor cells. Replication-selective tumor-specific adenoviruses are being developed as novel anticancer therapies (11–14). In this context, an adenoviral vector that drives E1A and E1B genes under the hTERT promoter has been developed, termed Telomelysin or OBP-301 (15). OBP-301 can replicate in and lyse only cancer cells but not normal cells, and its strong cytotoxic activity were shown in a variety human cancer cells (15–17). Also, OBP-301-mediated oncolysis induces uric acid production as a danger signal and stimulates CTL activity via proteasome activator up-regulation (18).

The infection efficiency of recombinant adenoviral vectors varies widely depending on the expression of the primary receptor, the coxsackie adenovirus receptor (CAR); the secondary receptors, integrin $\alpha_V\beta_3$ and integrin $\alpha_V\beta_5$; and the tertiary receptor, heparan sulfate glycosaminoglycans (HSG; refs. 19, 20). The first step is the attachment of

Received 7/1/08; revised 9/26/08; accepted 10/23/08.

Grant support: Japanese Ministry of Health, Labour and Welfare grant-in-aid KH33332 (K. Kawakami).

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doi:10.1158/1535-7163.MCT-08-0620

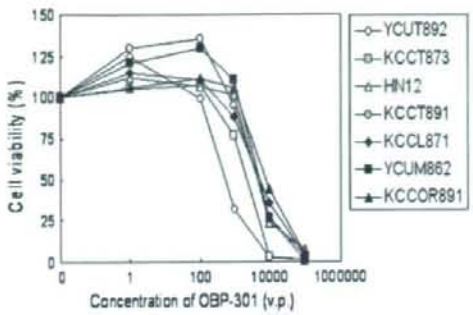

Figure 1. Sensitivity of SCCHN cells to OBP-301 *in vitro*. Cytotoxic activity of OBP-301 on 13 SCCHN cell lines was evaluated by XTT assay. Cells were cultured with various concentrations of OBP-301 (0-100,000 vp/mL). Mean ± SD of quadruplicate determinations. The assay was repeated three times.

the virus to the cell surface through CAR (20). Following attachment, the internalization of the virus into cells occurs through the integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$ that are expressed in most cell types (19).

Previously, OBP-301 has been reported to induce cell death of human non-small cell lung, colorectal, and prostate cancers *in vitro* and *in vivo* (15, 17). The present study investigates the cytotoxic activity of OBP-301 in 13 SCCHN cell lines and the association between cytotoxic activity and adenoviral receptor expression. We also assessed the *in vivo* antitumor activity and toxicity and tolerability of OBP-301 in an athymic nude mouse model with KCCT873 SCCHN tumors.

Materials and Methods

Adenovirus

The recombinant replication-selective, tumor-specific adenoviral vector OBP-301 was provided by Oncolys Biopharma. The hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (15). The virus particle (vp) titer-to-infection titer (plaque-forming units) ratios were 110:3.

Cells

The human non-small cell lung cancer cell line H1299 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L HEPES (Nacalai Tesuque), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesuque). The SCCHN cell line HN12 was grown in MEM containing 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The SCCHN cell lines YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, KCCOR891, YCUL891, YCUM911, YCUMS861, YCUT891, 012SCC, and Wmm-SCC (21) were cultured in RPMI 1640 containing 10% fetal bovine serum, 1 mmol/L HEPES, 100 μg/mL penicillin, and 100 μg/mL streptomycin.

Cell Viability Assay

The XTT assay was done to measure cell viability. Briefly, cells were plated on 96-well plates at 1×10^3 per well 24 h before viral infection. Cells were then infected with 1 to 1×10^5 multiplicity of infection (vp) of OBP-301 and further cultured for 120 h. Cell viability was determined using the Cell Proliferation Kit II (Roche Diagnostics) according to the protocol provided by the manufacturer.

Flow Cytometry

Cells (1×10^5) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Cell Signaling Solution), anti-integrin $\alpha_V\beta_3$ (Chemicon), anti-integrin $\alpha_V\beta_5$ (Chemicon), or anti-heparan sulfate (Seikagaku) for 60 min at 4°C, incubated with FITC-conjugated goat anti-mouse IgG secondary antibody (Chemicon), and analyzed by the FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Control cells were incubated with anti-mouse IgG primary antibody (BD Bioscience) and FITC-conjugated goat anti-mouse IgG secondary antibody. G-means were calculated by the following formula: (G-means of antibody-treated cells) - (G-means of control cells). Correlation coefficients were obtained between the expression levels of CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, HSG, and the ID_{50} of OBP-301 in 7 SCCHN cell lines.

Quantitative Real-time PCR Analysis

Total RNA from cultured cells was obtained using the RNeasy Mini kit (Qiagen). Total RNA (~0.1 μg) was used for reverse transcription. Reverse transcription was done at 22°C for 10 min and then at 42°C for 20 min. The *hTERT* mRNA copy number was determined by real-time quantitative reverse transcription-PCR using a LightCycler instrument and a LightCycler DNA TeloTAGGG kit (Roche Diagnostics). PCR amplification was run with activation at 95°C for 15 s, annealing at 58°C for 10 s, and extension at 72°C for 9 s.

Athymic Nude Mouse Models of Human Head and Neck Cancer

Five- to 6-week-old female athymic nude mice (BALB/c *nu/nu*) were obtained from SLC. Animal care was in

Table 1. Cytotoxic activity of adenoviral receptors on head and neck cancer cell lines

Cell line	Origin	ID_{50} (vp)*
YCUT892	Tongue	759
KCCT873	Tongue	4,279
HN12	Lymph node	6,943
KCCT891	Hypopharynx	7,025
YCUM862	Oropharynx	7,512
KCCL871	Larynx	7,599
KCCOR891	Oral floor	9,204
YCUL891	Larynx	ND
YCUM911	Oropharynx	ND
YCUMS861	Maxillary sinus	ND
YCUT891	Tongue	ND
012SCC	Unknown	ND
Wmm-SCC	Unknown	ND

Abbreviation: ND, not done.
*ID_{50}, infection dose of OBP-301 at which 50% inhibition of cell viability is observed compared with untreated cells.

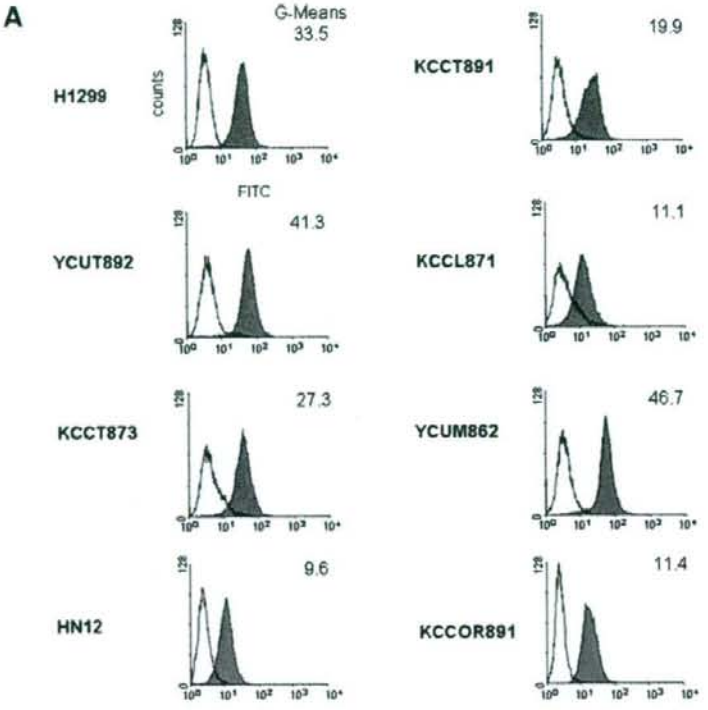

Figure 2. Expression of the CAR in SCCHN cell lines. **A,** cells were incubated with mouse monoclonal anti-CAR (RmcB) followed by detection with FITC-labeled secondary antibody. *Gray histogram,* staining with anti-CAR antibody treatment. H1299 human lung cancer cells were used as a positive control. **B,** correlation between CAR expression in SCCHN cells and the ID_{50} of OBP-301 for these cells. **C,** correlation between integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ and HSG expression in SCCHN cells and the ID_{50} of OBP-301 for these cells. The experiment was repeated three times.

C

Cell line	$\alpha V\beta 3$	$\alpha V\beta 5$	HSG
H1299	5.16	9.6	27.1
YCUT892	0.5	7.7	102.4
KCCT873	31.1	10.8	9.6
HN12	1.9	4.4	7.0
KCCT891	1.6	5.5	7.7
KCCL871	2.9	4.6	105.4
YCUM862	1.5	1.3	20.7
KCCOR891	2.0	8.8	156.5
correlation coefficient	**-0.2**	**-0.3**	**0.1**

accordance with the guidelines of the Kyoto University School of Medicine. A SCCHN model was established in nude mice by s.c. injection of KCCT873 tumor cells (5×10^6) in 150 μL PBS into the flank. Palpable tumors developed within 3 to 4 days. Tumors were measured by vernier calipers. Six to 7 mice were used for each group.

Toxicity Assessment

Blood samples and organs were collected from athymic nude mice at day 10 or 17 after i.t. administration of OBP-301 (3×10^{10} vp/d for days 5-9). Organs from the experimental animals were fixed in 10% formalin, and 5 μm tissue sections were prepared and stained with H&E.

Statistical Analysis

Tumor volume on a given day was calculated by the following formula: (length of the tumor) × (width of the tumor)^2 / 2. The statistical significance of tumor regression was calculated by the Student's *t* test.

Results

Cytotoxic Activity of OBP-301 to Various SCCHN Cell Lines

We first examined the effect of OBP-301 infection on the viability of SCCHN cell lines assessed by the XTT assay.

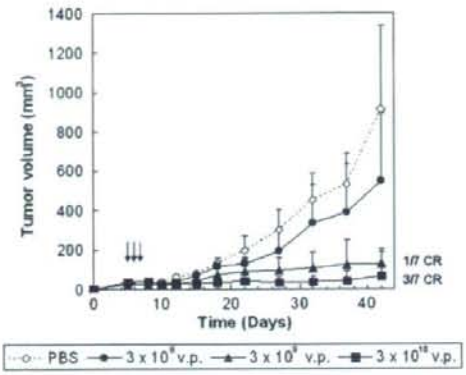

Figure 3. OBP-301 acted in a dose-dependent manner in KCCT873 tumor-bearing mice. Athymic nude mice received s.c. implantation of 5×10^6 KCCT873 cells on day 0. Animals then received injections of OBP-301 at the doses of 3×10^8 (●), 3×10^9 (▲), or 3×10^{10} vp (■) on days 5 to 7 (total of three injections). Each group had 7 animals, and the injection volume was 30 μL in each tumor. *Arrows*, day of injections. *Bars*, SD. The experiment was repeated two times.

Because OBP-301 showed slightly cytotoxic activity against 7 of 13 cell lines at a dose of 1,833 vp (50 plaque-forming units/cell), we assessed the ID_{50} of OBP-301 using these 7 SCCHN cell lines (Fig. 1; Table 1). As shown in Table 1, OBP-301 shows modest to strong cytotoxic activity in the 7 cell lines tested, with the ID_{50} varying from 759 to 9,204 vp. The cytotoxic activity of OBP-301 in these cell lines shows dose dependence (Fig. 1). YCUT892 cells were most sensitive to OBP-301 followed by KCCT873, HN12, KCCT891, YCUM862, KCCL871, and KCCOR871 cells, suggesting that 2 of the SCCHN cell lines are most sensitive to OBP-301.

Expression of Adenovirus Receptors in SCCHN Cell Lines

Because the cytotoxic activity of OBP-301 was anticipated to be correlated with efficiency of adenoviral infection through CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, or HSG receptors (22), we then assessed the expression levels of CAR on SCCHN cells using flow cytometry. As shown in Fig. 2A, all 7 SCCHN cell lines have been found to express detectable levels of CAR. However, as shown in Fig. 2B, correlation between the cytotoxic activity of OBP-301 and the expression level of CAR was not significant (correlation coefficient = −0.5). In addition, we assessed the expression of integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, and HSG in SCCHN cell lines; however, the expression levels were not correlated with cytotoxic activity of OBP-301 (Fig. 2C). We also assessed *hTERT* expression using the quantitative PCR method and found that all of the SCCHN cell lines express detectable levels of *hTERT* mRNA; however, there was no correlation between the expression levels and ID_{50} of OBP-301 in these cells (data not shown). These results suggest that, although 7 of 13 SCCHN cell lines are sensitive to OBP-301, its ability to enter the cell is not necessarily correlated with the degree of cytotoxicity.

Antitumor Effect of OBP-301 in SCCHN-Bearing Animals

To assess the antitumor effect of OBP-301 in the animal model of human SCCHN, KCCT873 cells were implanted s.c. in athymic nude mice (6, 23) to examine the effect of OBP-301 at a variety of dosages *in vivo*. Mice received i.t. injections of OBP-301 at 3×10^8, 3×10^9, or 3×10^{10} vp for 3 days from days 5 to 7 after tumor implantation (Fig. 3). Tumors grew to mean tumor volume of 32.2 ± 4.5 mm^3 at day 5. As shown in Fig. 3, the 3×10^8 dose of OBP-301 treatment was less effective against KCCT873 tumor growth. The mean tumor volume was 549 mm^3 on day 42, which is comparable with control tumor volume (910 mm^3). Higher doses of OBP-301 led to superior antitumor activity. The mean tumor volume of treated tumors was 130 mm^3 at 3×10^9 vp and 67 mm^3 at 3×10^{10} vp, which is significantly smaller compared with the control tumor at day 42 ($P < 0.0001$). Remarkably, in addition to a 93% inhibition in tumor volume in mice receiving a 3×10^{10} vp dosage, 3 of 7 tumors completely disappeared by day 37, which persisted through day 42. These results suggest that OBP-301 shows a remarkable antitumor effect in a dose-dependent manner in KCCT873 SCCHN tumors. Based on these findings, OBP-301 at a dosage of 3×10^{10} vp per injection shows the maximum tumor reduction effect.

Optimization of OBP-301 Injection Times in KCCT873 SCCHN Tumors

We next evaluated the treatment schedule of OBP-301 in s.c. xenografted KCCT873 tumor-bearing mice. Mice were treated i.t. with OBP-301 for 1, 3, or 5 subsequent days. The

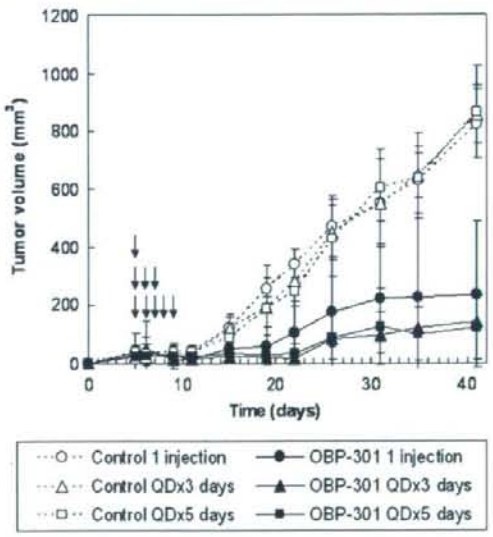

Figure 4. Regression of KCCT873 tumors by i.t. treatment of OBP-301. Athymic nude mice receiving s.c. KCCT873 implantation were treated with OBP-301 (3×10^{10} vp) for 1 (●), 3 (▲), or 5 (■) days. Injections were made on consecutive days (*QD*). Each group had 6 animals, and the injection volume was 30 μL in each tumor. *Arrows*, day of injections. *Bars*, SD. The experiment was repeated two times.

OBP-301 treatment started on day 5 (mean tumor volume, 31.4 ± 7.3 mm^3), as palpable tumors developed within 3 to 4 days. As shown in Fig. 4, i.t. administration of OBP-301 showed considerable antitumor activity in all groups. The mean tumor volume of animals receiving a one-time injection was 236 mm^3 at day 41, 71% smaller than excipient-only injected control tumors (823 mm^3; $P < 0.0001$). Interestingly, three or five injections of OBP-301 treatment showed superior antitumor activity. The mean tumor volume of animals in the group receiving a three-time treatment was 142 mm^3 at day 41, 83% smaller than control tumors (856 mm^3; $P < 0.0001$). Three of 6 tumors had completely regressed by day 27; however, later on, all of the tumors appeared and slowly started to grow again by day 41. Animals receiving OBP-301 for 5 days showed superior tumor response, including complete disappearance of tumors in 2 of 6 mice through day 41. The mean tumor volume measured on day 41 (121 mm^3) was 86% smaller than control tumors (863 mm^3; $P < 0.0001$). These results suggest that three- and five-time injections of OBP-301 treatment were equally effective in KCCT873 SCCHN tumor reduction.

Toxicity Profile in Mice Treated with OBP-301

Finally, to assess the toxicity and safety profile of OBP-301 treatment, blood and major organs including heart, liver, lung, kidney, and spleen were collected from KCCT873 tumor-bearing athymic nude mice receiving i.t. OBP-301 (3×10^{10} vp/d for 5 days) on day 10 or 17 after tumor implantation. As shown in Table 2, a blood serum chemistry analysis showed no remarkable changes in any variable in all the mice tested, except for a minor elevation of creatinine phosphokinase and aspartate aminotransferase in the OBP-301 treatment group. Similarly, no pathologic alterations were observed in any of the organs tested (data not shown). Although a slight necrosis was observed in livers from mice treated with i.t. OBP-301, all other organs from untreated control and OBP-301-treated mice did not show any evidence of toxicity. The result that all the treated mice tolerated therapy very well without any behavioral changes or toxicities in blood and pathology suggests that OBP-301 treatment leads to considerable antitumor activity without unwanted safety or toxicity issues.

Discussion

Although it has been reported that OBP-301 showed a strong anticancer activity in colorectal, prostate, and non-small cell lung cancer *in vitro* and *in vivo*, the effect of OBP-301 in SCCHN has not been pursued (17, 18, 24). Therefore, in this study, we planned to assess the detailed antitumor and toxicity profile of OBP-301 in an animal model of SCCHN. OBP-301 induces cell death in 7 of 13 cell lines *in vitro* and shows dramatic antitumor effects in an animal model bearing KCCT873 tumors without significant toxicity.

OBP-301 showed cytotoxic activity in 7 of 13 SCCHN cell lines. Because the effect of OBP-301 against SCCHN cell lines was limited compared with that previously shown against human non-small cell lung, colorectal, and prostate cancer cell lines (14, 17), we hypothesized that the limitation came from the lower viral infection rate. However, it is of interest to note that the expression levels of adenoviral receptors including CAR, integrins, and HSG are comparable between SCCHN and non-small cell lung cancer H1299 cell lines (Fig. 2; data not shown). In addition, we did not find a significant correlation between *hTERT* mRNA expression and the cytotoxic activity of OBP-301. These results suggest that various factors such as replication speed of viruses and the existence of unknown receptors might be involved in the cytotoxic activity of OBP-301.

The i.t. three- or five-time administration of OBP-301 dramatically inhibited the growth of KCCT873 tumors *in vivo*. The antitumor effect was actually superior to what we expected from our *in vitro* results. Previously, we reported that adenovirus present in blood of mice exists for at least 1 week after i.t. treatment with OBP-301 (15, 17), and i.t. OBP-301 showed antitumor effects both in the injected primary tumor site and in tumors located at distant sites (17). From these results, it is conceivable that OBP-301 attacked the xenografted KCCT873 tumor over and over through the bloodstream for at least 1 week after injection.

Table 2. Changes in blood serum chemistry of mice receiving OBP-301 treatment

Profile	Untreated control	Day 10*		Day 17*	
		PBS	OBP-301	PBS	OBP-301
Sodium (mEq/L)	156	153	151	157	156
Potassium (mEq/L)	7.5	7.3	9.3	8.7	8.2
Creatinine phosphokinase (units/L)	4,007	6,895	8,790	4,907	6,508
Lactate dehydrogenase (units/L)	2,153	3,197	3,158	2,600	2,740
Aspartate aminotransferase (units/L)	195	274	445	349	536
Alanine aminotransferase (units/L)	38	48	64	62	71
Bilirubin (mg/dL)	0.1	0.1	0.1	0.1	0.1
Creatinine (mg/dL)	0.16	0.13	0.13	0.14	0.13

NOTE: Data are mean blood samples from 3 animals in each group.
*Blood samples were collected from athymic nude mice receiving five i.t. injections of OBP-301 (days 5-9).

It has been reported that oncolytic virus replication induces tumor-specific immune responses by stimulating uric acid production as a danger signal as well as accelerating tumor antigen cleaved by IFN-γ-inducible PA28 expression (18). Additionally, because it has been shown that telomerase is active in ~80% to 90% of SCCHN tumor tissues as assessed by immunohistochemistry (25), we speculate that SCCHN cancer preferentially responds to OBP-301 treatment. These results may be the reason why the antitumor activity of OBP-301 is more profound in KCCT873 tumors than expected from our *in vitro* results. Therefore, the strong anticancer effect shown in these animal studies suggests that OBP-301 could be an attractive agent to accomplish an *in situ* radical cure of SCCHN patients.

Although chemoradiotherapy, radiotherapy plus concurrent chemotherapy, has become the standard care for patients with unresectable SCCHN and organ preservation (26, 27), it has recently been reported that cisplatin and fluorouracil with docetaxel plus chemoradiotherapy has a greater effect (28). Because our previous study showed that OBP-401 containing a green fluorescent protein gene for monitoring viral replication (TelomeScan) showed enhanced antitumor efficacy in an *in vivo* human lung cancer model when given in combination with docetaxel, it is possible that combination of OBP-301 with conventional chemotherapy may be a powerful regimen for the treatment of SCCHN in the clinic (29). Additionally, as SCCHN is easily acceptable site for direct injection (6) and i.t. OBP-301 was emerged strong antitumor effect in the xenografted KCCT873 tumor, the i.t. OBP-301 may be a new tool for the treatment of head and neck cancer. Future directions of clinical exploration with OBP-301 are still being considered. Utilization of OBP-301 via i.t. injection appears to be associated with modest activity, although clinical utility of local regional therapy is limited. Further exploration via intrahepatic arterial infusion or i.v. infusion awaits discovery of methods to improve OBP-301 activity.

Viral replication generally results in tissue destruction. In fact, interactions between adenovirus type 5 with CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, or HSG and the fiber shaft of adenovirus type 5 are known to be involved in accumulation in the liver of mice and cynomolgus monkeys when administered (30–33). In this study, a significant therapeutic effect of i.t. OBP-301 treatment was achieved without any significant liver toxicity. Histologic analyses in the brain, lung, heart, kidney, and spleen showed no toxicity profile. Oncolytic viruses have been developed as anticancer agents because controlled replication in the tumors causes selective killing of tumor cells and minimizes the effect on normal cells (34). Thus, the current results are consistent with the mechanism of action of virotherapy with oncolytic viruses.

Notably, a phase I study of OBP-301 has been initiated in the United States to test the safety and tolerability of OBP-301 in patients with various types of progressive solid cancer including SCCHN. Results from current clinical trials may further show additional information on its safety and efficacy. As for the clinical use of OBP-301 in SCCHN, the preliminary information obtained from our study is, based on the present results, considered to be useful for the planning of future clinical trials.

In conclusion, this study clearly shows that OBP-301 has remarkable *in vivo* anticancer effects against SCCHN. These findings suggest that the replication-selective oncolytic virus provides a new platform for treating patients with human head and neck cancer.

Disclosure of Potential Conflicts of Interest

Y. Urata and D. Ichimaru: employees of Oncolys Biopharma Inc. T. Fujiwara: consultant to Oncolys Biopharma Inc. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Hisashi Urushihara and Ritsuko Asai for technical support.

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Review Article

Understanding and exploiting *hTERT* promoter regulation for diagnosis and treatment of human cancers

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(Received March 18, 2008/Revised April 23, 2008/Accepted April 23, 2008/Online publication July 29, 2008)

Telomerase activation is a critical step for human carcinogenesis through the maintenance of telomeres, but the activation mechanism during carcinogenesis remains unclear. Transcriptional regulation of the human telomerase reverse transcriptase (*hTERT*) gene is the major mechanism for cancer-specific activation of telomerase, and a number of factors have been identified to directly or indirectly regulate the *hTERT* promoter, including cellular transcriptional activators (c-Myc, Sp1, HIF-1, AP2, ER, Ets, etc.) as well as the repressors, most of which comprise tumor suppressor gene products, such as p53, WT1, and Menin. Nevertheless, none of them can clearly account for the cancer specificity of hTERT expression. The chromatin structure via the DNA methylation or modulation of nucleosome histones has recently been suggested to be important for regulation of the *hTERT* promoter. DNA unmethylation or histone methylation around the transcription start site of the *hTERT* promoter triggers the recruitment of histone acetyltransferase (HAT) activity, allowing *hTERT* transcription. These facts prompted us to apply these regulatory mechanisms to cancer diagnostics and therapeutics. Telomerase-specific replicative adenovirus (Telomelysin, OBP-301), in which *E1A* and *E1B* genes are driven by the *hTERT* promoter, has been developed as an oncolytic virus that replicates specifically in cancer cells and causes cell death via viral toxicity. Direct administration of Telomelysin was proved to effectively eradicate solid tumors *in vivo*, without apparent adverse effects. Clinical trials using Telomelysin for cancer patients with progressive stages are currently ongoing. Furthermore, we incorporated green fluorescent protein gene (*GFP*) into Telomelysin (TelomeScan, OBP-401). Administration of TelomeScan into the primary tumor enabled the visualization of cancer cells under the cooled charged-coupled device (CCD) camera, not only in primary tumors but also the metastatic foci. This technology can be applied to intraoperative imaging of metastatic lymphnodes. Thus, we found novel tools for cancer diagnostics and therapeutics by utilizing the *hTERT* promoter. (*Cancer Sci* 2008; 99: 1528–1538)

In the past decade, research in the field of telomerases has progressed tremendously, especially in relation to cellular immortality and carcinogenesis. Telomerase activation is observed in approximately 90% of human cancers, irrespective of tumor type, while most normal tissues contain inactivated telomerase.[1] The role and timing of telomerase activation in carcinogenesis has been revealed by telomerase-knockout mouse studies.[2,3] Significant telomere erosions and age- and generation-dependent increases in cytogenic abnormalities are exhibited in telomerase-knockout mice, providing evidence that telomere dysfunction with critically short telomeres causes genomic instability.[2] This concept is further supported by studies using telomerase–/– p53–/– double-knockout mice.[3] These mouse cells demonstrate high levels of genomic instability, exemplified by increases in both formation of dicentric chromosomes and susceptibility to oncogenic transformation. These mice exhibit significantly decreased tumor latency and overall survival. Thus, in the absence of genome checkpoint functions, telomere dysfunction accelerates genomic instability, facilitating cancer initiation.[4] According to this concept, the genomic instability caused by telomere dysfunction occurs in the early stages of carcinogenesis, before telomerase activation. Subsequently, telomeres in these initiated cells undergo further progressive shortening, generating rampant chromosomal instability and threatening cell survival. Telomerase activation necessarily occurs at this stage to stabilize the genome and confer unlimited proliferative capacity upon the emerging and evolving cancer cell. In other words, cells that have acquired telomerase activity can obtain the capacity for cancer progression. Eventually, most cancer cells exhibit telomerase activity.

This cancer-specific telomerase activity provides an opportunity for us to utilize it for cancer diagnosis and treatment. Continuous effort has been made to uncover the molecular mechanisms of telomerase activation during carcinogenesis. The discovery of the telomerase subunit human telomerase reverse transcriptase (hTERT),[5,6] a catalytic subunit bearing the enzymatic activity of telomerase,[7,8] was the starting point for uncovering the cancer-specific activation of telomerase. Numerous studies have demonstrated that hTERT expression is highly specific to cancer cells and tightly associated with telomerase activity, while the other subunits are constitutively expressed both in normal and cancer cells.[9–12] Therefore, there is no doubt that hTERT expression plays a key role in cancer-specific telomerase activation. In this review article, we discuss the cancer-specific regulation of *hTERT* and its application for cancer diagnosis and treatment.

Cloning of the *hTERT* promoter and identification of the core promoter region containing *cis*- and *trans*-elements for cancer-specific transcription

In 1999 we and other groups successfully cloned the 5′-promoter region of the *hTERT* gene.[13–15] Transient expression assays using the 3.0 kb of the flanking sequences of the *hTERT* gene revealed that the transcriptional activity was up-regulated

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doi: 10.1111/j.1349-7006.2008.00878.x

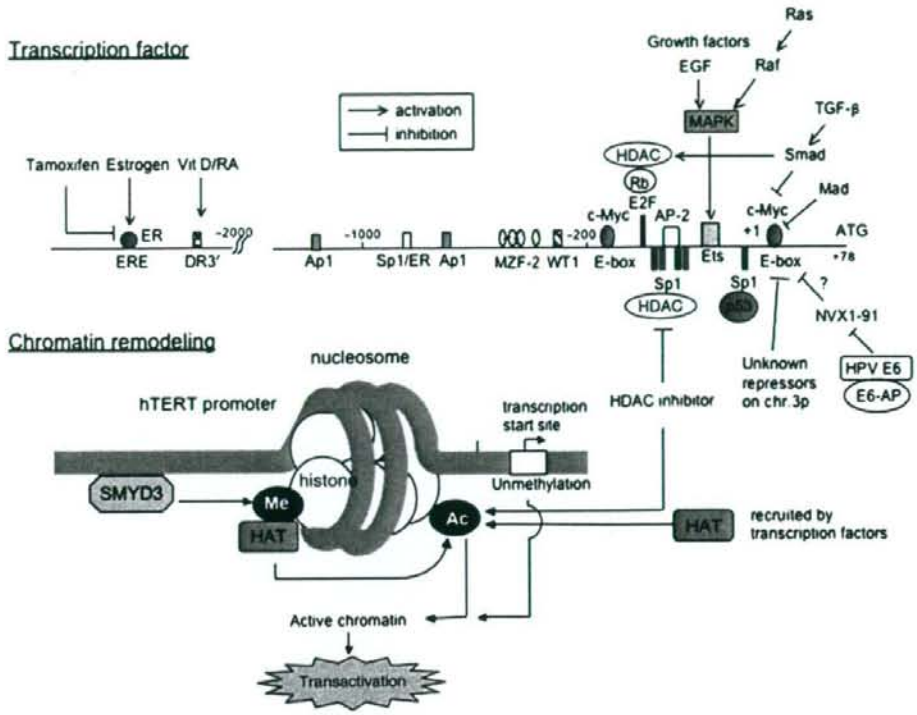

Fig. 1. Complex molecular mechanisms of transcriptional regulation of human telomerase reverse transcriptase (*hTERT*). Representative transcription factors and their upstream factors essential for *hTERT* regulation are shown in the upper panel. The sites on the promoter are not precisely in scale. +1 indicates the start site of transcription.[13] The proposed model of chromatin remodeling for the regulation of *hTERT* promoter is shown in the lower panel. Me, methylation of histone; Ac, acetylation of histone.

specifically in cancer cells, while it was silent in most normal cells.[13] Deletion analysis of the promoter identified the proximal 260 bp region functioning as the core promoter essential for cancer-specific transcriptional activation. Within the core promoter, several distinct transcription-binding sites are present; E-boxes (CACGTG) located at −165 and +44 (numbering based on the transcription start site determined by CapSite Hunting method[11]) are potential binding sites of basic helix-loop-helix zipper (bHLHZ) transcription factors encoded by the Myc family oncogenes. The existence of E-boxes on the *hTERT* promoter stirred telomerase researchers since c-Myc has been known to activate telomerase.[16] In fact, several groups confirmed that c-Myc binds to E-boxes on the *hTERT* promoter and activates the transcription[15–19] which established the scenario that c-Myc is a key regulator of *hTERT* transcription during carcinogenesis. However, several studies found that Myc and hTERT expression levels are not necessarily tightly correlated in some cancer cells.[20,21] Furthermore, it should be noted that most of these studies used overexpressed c-Myc for the luciferase reporter assay as well as recombinant c-Myc for the electrophoretic mobility shift assay (EMSA) to demonstrate binding to the E-boxes. Therefore, it remains unclear whether endogenous binding of c-Myc on the *hTERT* promoter plays a critical role in *hETRT* transcription *in vivo*, especially during carcinogenesis. Xu *et al.* reported the important finding that endogenous c-Myc binding to the E-boxes on the *hTERT* promoter was well correlated with the induction of *hTERT* in proliferating leukemic cells.[22] Nevertheless, it remains unclear whether up-regulation of *in vivo* binding of c-Myc to the *hTERT* promoter occurs during carcinogenesis and how critical it is for continuous hTERT expression in cancer.

Other characteristic sequences that exist on the *hTERT* promoter are the GC-boxes (GGGCGG), which are binding sites for zinc finger transcription factor Sp1. There are at least five GC-boxes within the core promoter of *hTERT*, proven by EMSA to bind Sp1.[22] Introduction of mutations in these GC-boxes significantly decreased the transcriptional activity of the promoter, while overexpression of Sp1 in cells that contain relatively low levels of endogenous Sp1 enhanced the promoter activity.[17] In particular, the *hTERT* core promoter activity was almost completely diminished by introducing mutations in all five GC-boxes, while mutation in one site moderately decreased it. Therefore, the GC-boxes function synergistically to maintain the promoter activity of *hTERT*. However, Sp1 is ubiquitously expressed in a wide range of normal cells, and is not therefore a strong candidate to cause cancer-specific hTERT expression.

Overall, while the *hTERT* core promoter is highly specific to cancer cells, the key transcription factors identified are far from accounting for cancer-specific *hTERT* expression.

Critical factors that regulate *hTERT* transcription

A number of factors that regulate *hTERT* transcription have been identified to regulate the *hTERT* promoter. The representative regulators of *hTERT* promoter with regard to the clinical aspects are shown in Fig. 1.

Cellular transcription factors. Several transcription factors, as well as c-Myc and Sp1, have been identified to regulate the *hTERT* promoter. Activating Enhancer-binding Protein-2 (AP-2) was recently identified as a transcriptional activator of the *hTERT* promoter[23] and, of particular interest, it exhibited tumor-specific

binding to the core promoter region. Although this study examined only one tumor type (lung cancer), this may partly explain tumor-specific *hTERT* transcription.

Hypoxia-inducible factor-1 (HIF-1), a key regulator of O_2 homeostasis, regulates the expression of several genes linked to angiogenesis and energy metabolism. The presence of putative HIF-1 binding sites on the *hTERT* promoter prompted us to examine the involvement of HIF-1 in regulation of *hTERT* in tumor hypoxia: we found that hypoxia activated hTERT mRNA in cancer cells *in vitro*.[24,25] Luciferase reporter assays revealed that *hTERT* transcription was significantly activated in hypoxia and by HIF-1α overexpression, and that the two putative HIF-1 binding sites within the core promoter are responsible for this activation. The chromatin immunoprecipitation assay identified specific binding of HIF-1α to these sites, which was enhanced in hypoxia. siRNA inhibition of HIF1-α abrogated hypoxia-induced hTERT mRNA expression. Thus, hypoxia activates telomerase mainly via transcriptional activation of *hTERT*, and HIF-1 plays a critical role as a transcription factor. In contrast to these findings, Koshiji *et al.* observed that HIF-1 inhibited *hTERT* expression in colon cancer cells.[26] In this study, they demonstrated that HIF-1 induces cell-cycle arrest even in the absence of hypoxia by functionally counteracting Myc. Eventually, HIF-1 down-regulates Myc-activated genes including *hTERT*. The reasons for this discrepancy remain unclear, but experimental conditions, such as the concentration of oxygen and constitutive levels of HIF-1 in cell types used, may significantly affect the results. A recent study underscored the importance of HIF-2 in regulating *hTERT* promoter.[27] While HIF2-α enhances *hTERT* expression in renal-cell carcinoma, it represses *hTERT* transcription in glioma cells, adding a further layer of complexity to the relationship between hypoxia and telomerase activity.

We also found the transcription activator protein AP-1 to function as a transcriptional repressor.[28] There are two AP-1 sites (at –1655 and –718) within the 2.0 kb promoter of *hTERT*. EMSA revealed that JunD is the major factor binding to them, which was further supported by chromatin immunoprecipitation (ChIP) assay *in vivo*. Overexpression of Jun family members with c-fos significantly reduced the promoter activity while mutation of AP-1 sites increased it. Of particular interest is the observation that AP-1 had no effect on the mouse *TERT* (*mTERT*) promoter although it has similar binding sites for AP-1. Since *mTERT* is constitutively expressed both in tumor and normal cells, this species-specific function of AP-1 in *TERT* expression may in part help explain the difference in telomerase activity between normal human and mouse cells.

Hormones. Hormonal regulation of *hTERT* and the molecular mechanisms involved have been analyzed most extensively in relation to estrogen. We and other groups found that estrogen activates *hTERT* transcription via binding of ligand-activated estrogen receptor-α (ERα) to the estrogen-responsible element (ERE) in the *hTERT* promoter.[29,30] ER-Sp1 half-sites located downstream of the ERE similarly function as *cis*-acting elements in response to estrogen stimulation. Estrogen also activates *hTERT* expression via post-transcriptional mechanisms with the stimulation of nuclear accumulation of hTERT via its phosphorylation, which is mediated by Akt signaling.[31] Tamoxifen, a selective estrogen receptor modulator, also regulates *hTERT* expression in a cell-type-specific manner;[32] tamoxifen inhibits the growth of breast cancer cells, as well as hTERT mRNA expression in the presence of estrogen (E2), antagonizing the E2 effects, in which the ERE on the promoter is involved. In contrast, tamoxifen stimulated the growth of endometrial cancer cells and activated hTERT mRNA expression in the absence or presence of E2, exhibiting estrogen-agonistic action, in which MAP kinase signaling pathways are involved. Androgen was also shown to activate hTERT mRNA in androgen-sensitive prostate cancer cells but this regulation was not due to *hTERT* promoter activation.[33] Progesterone exerts diverse effects on hTERT mRNA expression in a time-dependent manner in progesterone-receptor-positive breast cancer cells;[34] in the short term, it activates *hTERT* transcription, but prolonged exposure to progesterone antagonizes estrogen and inihibits *hTERT* transcription. Interestingly, both short- and long-term regulation is mediated via the MAP kinase signaling pathway.

Cytokines. Telomerase activation is known to be tightly associated with cell proliferation, which suggests that growth signaling might directly regulate hTERT expression.[35–37] We established an *in vitro* model in which telomerase activity can easily be induced upon stimulation of EGF in EGF-receptor-positive cancer cells.[38] Luciferase reporter assays revealed that EGF activates the *hTERT* promoter: an Ets motif located in the core promoter of *hTERT* is responsible. Notably, MAP kinase signaling pathways mediate this regulation. A number of growth signals have been known to be mediated through MAP kinase pathway, with Ets factors playing critical roles as final mediators regulating the target-gene expression. Therefore, EGF-mediated Ets-based *hTERT* transcription may be one representative pathway through which various growth signals are transduced to the *hTERT* promoter. This scenario can partly account for telomerase activation associated with cell proliferation.

TGF-β is a representative cytokine that represses *hTERT* transcription.[39] The mechanisms through which TGF-β down-regulates *hTERT* transcription are controversial: while some studies demonstrated that TGF-β repressed *hTERT* transcription via indirect down-regulation of c-Myc expression,[40,41] others reported direct interaction of Smad3 and c-Myc disturbing c-Myc activity.[42] Another study identified several negative regulatory factors for *hTERT* by means of gene screening using enhanced retroviral mutagenesis (ERM) and found that Smad interacting protein-1 (SIP1) is a repressor for *hTERT*, possibly mediating TGF-β signals.[43] A more recent study using siRNA inhibition of the Smad family confirmed that TGF-β-mediated repression of *hTERT* transcription is largely mediated through Smad3, not Smad1 or Smad2.[44] However, this study found no role for E-boxes in this repression, but found four E2F-binding sites within the proximal promoter of *hTERT* to be responsible, based on the data that mutation of these four sites reversed TGF-β-mediated repression of *hTERT* transcription. The transcriptional activity of E2F family members is regulated by interactions with pocket proteins (Rb, p107, p130) that recruit histone deacetylase (HDAC) proteins to repress target genes. Interestingly, overexpression of the dominant negative E2F gene lacking the ability to bind pocket protein (Rb, p107, p130) and to recruit HDAC significantly abrogated TGF-β-mediated repression of *hTERT* transcription. Furthermore, trichostatine A (TSA), a HDAC inhibitor, completely reversed the inhibitory effect of TGF-β. These findings highlight E2F and HDAC as central mediators of TGF-β-mediated repression of *hTERT* transcription. The involvement of HDAC in *hTERT* transcription is also discussed below.

Oncogenes. High-risk human papillomaviruses (HPV) are representative oncoviruses whose E7 protein can bind to Rb and alleviate repression of E2F-dependent target genes, thereby allowing rapid progression into S phase[45] while E6 protein facilitates the degradation of p53 through the actions of E6-associated protein (E6-AP), which results in the abrogation of the G_1/S and G_2/M checkpoints.[46–48] The initial study found that telomerase is activated in keratinocytes stably expressing HPV16 E6.[49] Since E6 had been known to activate c-Myc expression[50] it seemed likely that E6 activates *hTERT* transcription via up-regulating c-Myc. However, subsequent studies confirmed that high-risk HPV E6 activates *hTERT* transcription but is not associated with up-regulation of c-Myc.[51–53] Several studies found that *hTERT* transactivation by HPV16 E6 correlates with its ability to bind E6-AP.[54] A correlation between E6-AP binding and *hTERT* induction prompted the search for possible targets of

doi: 10.1111/j.1349-7006.2008.00878.x

the E6/E6-AP complex by a yeast two-hybrid screen, which identified a transcriptional repressor known as NFX1 that binds to 48-bp sequences surrounding the proximal E-box on the *hTERT* promoter.[54] It is supposed that the E6/E6-AP complex induces hTERT expression by destabilizing NFX-1. In support of this, decreased expression of NFX1 using siRNAs was sufficient to induce *hTERT* expression and telomerase activity in primary human epithelial cells.

Some human oncoproteins specifically activate *hTERT* promoter. In hTERT-negative normal cells, HER2/Neu signals (by overexpressing oncogenic HER2/Neu mutant) alone failed to activate the endogeneous *hTERT* expression.[55] However, coexpression of HER2/Neu with one ETS family member (ER81) successfully activated *hTERT* expression in these cells. There are five putative binding core GGAA/T sites for ETS family in exon1 to intron1 of the *hTERT* gene, and ER81 specifically binds to two of them and activates *hTERT* promoter in cooperation with HER2/Neu signals. Notably, this activation was mediated via the ERK-MAP kinase pathway, in which upstream Ras and Raf-1 play critical roles. Thus, three prominent oncoproteins, HER2/Neu, Ras, and Raf, facilitate *hTERT* expression via an Ets family member in hTERT-negative normal cells.

Epigenetic regulation of *hTERT* transcription

The *hTERT* promoter contains a cluster of CpG sites, and many researchers therefore supposed its regulation to involve DNA methylation. Several groups examined the methylation status of these CpG sites on this promoter. It was initially expected that methylation of the *hTERT* promoter was associated with gene silencing; indeed, some groups showed such association.[56–58] However, other reports indicated no significant correlation between *hTERT* expression and methylation status either overall or at a specific site.[59,60] Furthermore, contradictory results have been reported: increased DNA methylation in the *hTERT* promoter was observed in hTERT-positive cancer cells while lack of methylation was found in normal hTERT-negative cells.[61] These unusual correlations between DNA methylation and hTERT expression in normal and cancer cells generated confusion among telomerase researchers. Recently, Zinn *et al.* aimed to clarify the discrepancies:[62] using bisulfite sequencing, they first identified that all telomerase-positive cancer cell lines examined retained alleles with little or no methylation around the transcription start site despite being densely methylated in more upstream regions. ChIP assay revealed that both active (acethyl-H3K9 and dimethyl-H3K4) and inactive (trimethyl-H3K9 and trimethyl-H3K27) chromatin marks are present across the *hTERT* promoter. Subsequent Chip-MSP (methylation-specific polymerase chain reaction [PCR]) assay identified that active chromatin mark DNA around the transcription start site was tightly associated with unmethlated DNA. These data suggest that the absence of methylation and the association with active chromatin marks around the transcription start site allow for the expression of *hTERT* (Fig. 1), indicating that the DNA methylation pattern of the *hTERT* promoter is consistent with the usual dynamics of gene expression.

Modification of nucleosome histones, including acetylation/deacetylation as well as methylation, is known to regulate chromatin structure and thereby affect gene transcription.[63] Roles for histone-modification-mediated chromatin remodeling in the regulation of *hTERT* transcription have been revealed (Fig. 1). We and other groups found that treatment with TSA induced significant elevation of hTERT mRNA expression and telomerase activity in normal cells, but not in cancer cells.[64,65] Transient expression assays revealed that TSA activates the *hTERT* promoter, for which the proximal core promoter was responsible. Overexpression of Sp1 enhanced responsiveness to TSA, and mutation of Sp1 sites but not c-Myc sites of the core promoter of *hTERT* abrogated this activation. Introduction of the dominant-negative form of the Sp family inhibited TSA activation. These results indicate that HDAC inhibitor activates the *hTERT* promoter in normal cells in an Sp1-dependent manner (Fig. 1). It is possible that endogeneous Sp1 interacts with HDAC and recruits it to the *hTERT* promoter[66] resulting in the deacetylation of nucleosome histones, leading to the repression of transcription. While Sp1 contributes to the transactivation of *hTERT* as a potent transcriptional activator[22] it might be involved in gene silencing of *hTERT* in normal cells, possibly by recruiting HDACs. Compelling evidence suggests that Sp1 interacts with a p300 coactivator possessing intrinsic histone acetyltransferase (HAT) activity.[67] Therefore, it is possible that Sp1 interacts with various factors that have HAT or HDAC activity, and that this switching explains the different actions of Sp1 on the *hTERT* promoter in normal and cancerous cells. The E-box binding activator c-Myc and repressor Mad1[21,22,68] which compete with each other for the common binding partner Max are also involved in histone-modification-mediated chromatin remodeling of the *hTERT* promoter. The endogenous c-Myc/Max complex to the *hTERT* promoter in proliferating leukemia cells was found to be associated with the acetylated histones, resulting in enhanced hTERT expression.[22] In contrast, the complex was replaced by the endogeneous Mad1/Max complex that was associated with deacetylated histones and decreased *hTERT* expression in differentiated status.

Recently, a role for histone methylation in *hTERT* regulation has also been demonstrated. Atkinson *et al.* observed that highly trimethylated H3-K4 was associated with the actively transcribed *hTERT* gene in telomerase-proficient tumor cells.[69] More recently, we reported the interesting finding that SET- and MYND-domain-containing protein-3 (SMYD3), a histone H3-K4-specific dimethyltransferase and trimethyltransferase, respectively, play critical roles in H3-K4 methylation of the *hTERT* promoter.[70] Of the various SET-domain-containing proteins, SMYD3 is unique because not only does it have methyltransferase activity but it also binds to a specific DNA sequence (CCCTCCC) in its target promoters, as do transcription factors. In fact, SMYD3 was confirmed to bind some of the CCCTCCC motifs within the core promoter of *hTERT* and activate *hTERT* transcription. Overexpression of *SYND3* induced hTERT mRNA expression in hTERT-negative normal and cancer cells. Disruption of SMYD3 binding motifs in the *hTERT* promoter led to significant reduction of transcription. Expectedly, siRNA-knockdown of *SMYD3* resulted in abolishment of H3-K4 trimethylation of the *hTERT* promoter in cancer cells; interestingly, this knockdown also led to defects in binding c-Myc and Sp1. Furthermore, histone H3 acetylation within the core promoter of *hTERT* was diminished by the *SMYD3*-knockdown. These data suggest a model in which SMYD3 binding to the *hTERT* promoter leads to increased H3 trimethylation, a critical event that recruits HAT and promotes Sp1 and c-Myc access to the *hTERT* promoter (Fig. 1). Thus, SMYD3-mediated trimethylation of H3-K4 may function as a licensing element for subsequent transcription-factor binding to the *hTERT* promoter, which may trigger further recruitment of HAT activity.

Identification of *hTERT* repressors

Recently, Lin *et al.*[43] identified several negative regulatory factors for *hTERT* by means of gene screening that used enhanced retroviral mutagenesis (ERM). They identified menin, SIP1, Mad1, hSIR2, and BRIT1 as candidates for the *hTERT* repressor, generating the idea that multiple tumor suppressors might involve telomerase repression, especially in normal cells. p53 was also shown to repress *hTERT* transcription in a Sp1-dependent manner.[71,72] It was proved that p53 can form a complex with Sp1, which disturbs the transcriptional activity of Sp1 and leads to transcriptional repression.[72] Several transcriptional repressors,

including Wilms' tumor 1 tumor suppressor (WT1) and myeloid-specific zinc finger protein-2 (MZF-2) are also known to repress *hTERT* transcription via binding to their specific sites on the promoter, although the mechanisms of repression remain unclear.[73,74] We also found that on combinatorial treatment with Vitamin D3 and 9-*cis*-retinoic acid, the heterodimer complex, vitamin D[3] receptor/retinoid X receptor (RXR), binds to the distal sites on the *hTERT* promoter and represses transcription.[75]

There has been an extensive search for telomerase repressors, one of which was based on microcell-mediated chromosome transfer.[76] Several normal human chromosomes, including chromosomes 3, 4, 6, 7, 10, and 17, have been shown to repress telomerase activity in some but not all cancer cells.[77–85] Horikawa *et al.* established a nice system to investigate an endogenous mechanism for telomerase repression using a telomerase-positive renal carcinoma cell line (RCC23) and telomerase-negative counterpart (RCC23 + 3) generated by transferring a normal chromosome 3 into RCC23 cells.[86] By comparing the molecular characteristics of these cells, they identified the E-box downstream of the transcription initiation site that was responsible for telomerase repressive mechanisms restored by normal chromosome 3 targets. They also found that the factors binding to the E-box, other than c-Myc/Mad or USF families, were involved in the transcriptional repression of *hTERT* although they remained to be cloned. This E-box-mediated repression functions in various types of normal human cells, while it is inactive in some, but not all, hTERT-positive cancer cells, providing evidence for an endogenous mechanism for *hTERT* transcriptional repression that becomes inactivated during carcinogenesis.

hTERT promoter for cancer therapeutics

***hTERT* promoter for cancer-specific transgene expression.** In the field of cancer gene therapy, the researchers have a great interest in efficiently expressing target genes in the tumor tissue while decreasing adverse effects in normal tissue. Control of gene expression via tissue- or cell-specific promoters has been tested extensively as a means of targeting transgene expression. Several promoters have been identified that are more active in particular tumor types than in the tissues from which they arise, and these promoters have been exploited to target transgene expression in tumors. These promoters include the tyrosinase gene promoter in melanomas,[87] the carcinoembryonic antigen promoter in colorectal and lung cancer,[88] the MUC1 promoter in breast cancer,[89] and the E2F promoter in cancers that carry a defective retinoblastoma gene.[90] However, while reports on these promoters suggest that achieving relatively tumor-specific transgene expression is possible, several limitations have also been revealed. First, most of these promoters are limited to specific tumor histologies and cannot be used universally in tumors of various origins. Second, most of these promoters are much weaker than commonly used viral promoters such as the CMV early promoter, the Rous sarcoma virus long-terminal repeat (RSV-LTR), and the SV40 early promoter. Consequently, their use is hampered by the problem of low expression.

The *hTERT* promoter is ideal to overcome the shortcoming of these promoters. Gu *et al.* first established the binary adenoviral system, which uses two adenoviral vectors to induce *Bax* gene expression.[91] One of these vectors contains a human *Bax* cDNA under the control of a minimal synthetic promoter comprising five Gal-4-binding sites and a TATA box, which is silent in 293 packaging cells, thus avoiding the toxic effects of the *Bax* gene on the 293 cells and allowing vector (Ad/GT-Bax) production. Expression of the *Bax* gene can be induced by coinfecting the Ad/GT-Bax virus with the second adenoviral vector in the binary system (Ad/PGK-GV16), which consists of a fusion protein comprising a Gal-4 DNA-binding domain and a VP 16 activation domain under the control of a constitutively active PGK promoter. Ad/PGK-GV16 is expected to produce VP16 with Gal-4 DNA binding domain preferentially in tumor cells and thereby induce *Bax* gene expression via interaction with Gal-4-binding sites. This binary infection system was reported to suppress tumor growth *in vitro* and *in vivo*. More simple vector systems to achieve cancer-specific transgene expression have been tried, in which several apoptosis-inducible genes such as *FADD*,[92,93] *caspace*[94,95] or suicide gene (human herpes simplex virus thymidine kinase (*HSVtk*) gene),[96] tumor-necrosis-factor-related apoptosis-inducing ligand gene (*TRAIL*),[97] or chemoattractant protein gene (*MCP-1*)[98] have been driven by the *hTERT* promoter in various tumor types. Most of these studies successfully demonstrated tumor-specific transgene expression *in vivo*, achieving long-term survival benefit and minimizing its expression in normal tissues following direct injection of the vectors and even with systemic injection. Systemic toxicity is one concern in this treatment modality because telomerase activity has been reported to exist in some normal cells, such as hematopoietic crypt and endometrial cells, most of which have high regenerative potentials. Gu *et al.* tested *hTERT*-promoter-driven transgene expression in human CD34(+) bone marrow progenitor cells and found very low *hTERT* promoter activity in these cells as well as no detectable change in blood-cell profiles under long-term observation.[99] Basically, the *hTERT* promoter activity in these normal cells with telomerase activity is much lower than that in cancer cells, and toxicity is expected to be minimized.

***hTERT* promoter for cancer-specific replication-competent adenovirus.** Despite these efforts, levels of transgene expression were insufficient to eradicate tumors, especially when vectors were systemically administrated. This is mainly due to the characteristics of adenoviral vectors used, in which the *E1* gene was deleted to inhibit replicative capacity. These nonreplicative vectors had limited distribution within the tumor mass even after direct intratumoral administration. To confer specificity of infection and increase viral spread to neighboring tumor cells, the use of replication-competent adenoviruses has become a reality. The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for the treatment of cancer. Many efforts have been made to realize cancer-specific adenoviral replication using a variety of gene promoters, including the prostate-specific antigen,[100] MUC1,[101] osteocalcin,[102] L-plastin,[103] midkine,[104] and *E2F-1* genes.[105] Unfortunately, these promoters have tissue-type specificity and exhibit transcriptional activity only in cells that express such tumor markers. Furthermore, the transcriptional activity is relatively low. We were prompted by these studies to use the *hTERT* promoter, hypothesizing that an adenovirus containing the *hTERT* promoter-driven *E1* genes could target a variety of tumors and kill them with high replicative capacity.

We developed a novel telomerase-dependent replicative adenovirus type 5 vector (Telomelysin, OBP-301) in which *E1A* and *E1B* genes, required for adenoviral replication, were transcribed under the *hTERT* promoter.[106] In most vectors that replicate under the transcriptional control of the *E1A* gene, *E1B* is driven by the endogeneous adenovirus *E1B* promoter. However, the insertion of internal ribosome entry site (IRES) between *E1A* and *E1B* improved the promoter specificity of *E1B* transcription. We selected the 455 bp-proximal promoter region of the *hTERT* gene to drive *E1A* and *E1B* genes because our previous experiments showed that this region exhibits the highest transcriptional activity, comparable to the proximal core promoter.[13] The construction of Telomelysin is shown in Fig. 2. Similar replicative adenoviruses controlled by the *hTERT* promoter have also been developed by other groups.[107–109]

In vitro replication assays revealed that Telomelysin induced selective expression of *E1A* and *E1B* in cancer cells, resulting in viral replication at 5–6 orders of magnitude by 3 days after infection, while it was attenuated by up to 2 orders of magnitude

doi: 10.1111/j.1349-7006.2008.00878.x

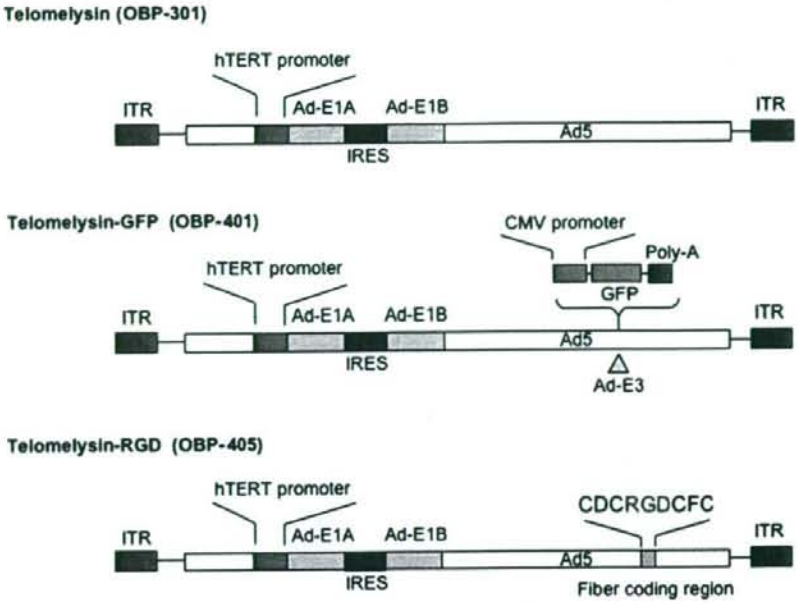

Fig. 2. Schematic DNA structures of telomerase-specific oncolytic viruses. Telomelysin (OBP-301) has *E1A* and *E1B* genes linked with an *IRES*, driven by the human telomerase reverse transcriptase (*hTERT*) promoter. A variant of OBP-301 was constructed that has the green fluorescent protein (*GFP*) gene at the *E3* region driven by *CMV* promoter (OBP-401). Another variant (OBP-405) has a mutant fiber containing the RGD peptide in the HI loop of the fiber knob.

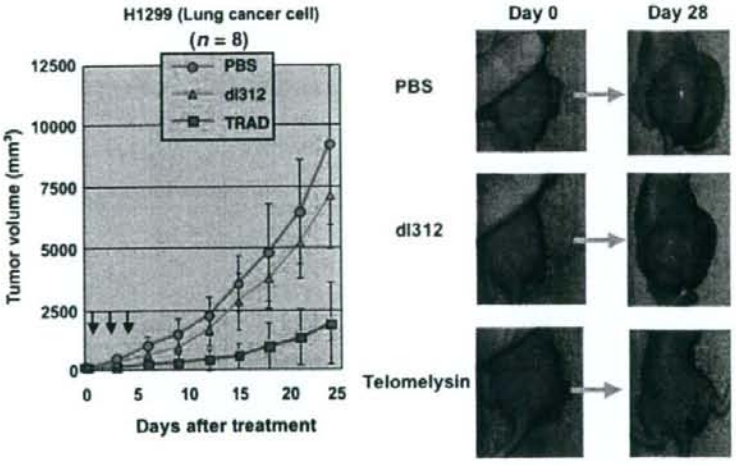

Fig. 3. *In vivo* effect of Telomelysin on tumorigenesis. Lung cancer H1299 cells were inoculated to the flank of nu/nμ mice. Mice bearing palpable tumors with a diameter of 5–6 mm received intratumoral injection of 10^7 PFU of Telomelysin or replication-deficient adenovirus (dL312) or phosphate-buffered saline (PBS) (mock treatment) on three consecutive days. The macroscopic appearances of H1299 tumors in nu/nμ mice at 0, 14, and 28 days after the treatment are shown. Note that the tumor growth was severely retarded by the treatment with Telomelysin. A modified version of this figure appeared in our original article.[106]

in cultured normal cells.[106,110] We confirmed that the transduction efficiency did not greatly differ in cancer and normal cells. Therefore, such difference in replication was considered to be due to the tumor specificity of Telomelysin. Since hTERT expression is observed broadly in a variety of tumor types, Telomelysin was expected to replicate in various cancer cells. Indeed, Telomelysin could efficiently kill head and neck, lung, esophageal, pancreatic, hepatic, prostate, and cervical cancers, as well as melanoma, sarcoma, and mesothelioma cells.[106,110]

The *in vivo* antitumor effect of Telomelysin was further investigated using mouse xenografts. Intratumoral injection of Telomelysin into inoculated tumors effectively retarded tumor growth and extended the survival of mice (Fig. 3). Telomelysin was also effective in progressive tumors with large tumor burden. When Telomelysin was directly injected to xenograft tumors after maximum growth, their size apparently decreased with the formation of massive ulceration at the site of injection.[106,110]

One technical merit for the use of replicative adenovirus is the unlimited replicative potential of virus over tumor mass. After tumor lysis due to viral toxicity, replicated viral particles can be released from tumors and spread to the whole body via blood or lymphatic flow[106] and finally replicate again at metastatic sites if they are telomerase-positive. Thus, in theory, Telomelysin

might have efficacy against not only primary lesions but also metastatic sites.

Administration of Telomelysin in combination with chemotherapeutic agents. To enhance the therapeutic potential of Telomelysin, efforts have been made to combine it with several chemotherapeutic agents. Combination with docetaxel, vinorelbine (Nevelbine), or SN38 (active metabolite of irinotecan) has been confirmed *in vitro* to enhance Telomelysin cytotoxicity in different organs including the lung, colon, esophagus, stomach, liver, and prostate.[111] Of particular interest were the synergistic effects of Telomelysin when it was administrated intratumorally to xenografts in combination with intraperitoneal administration of docetaxel. The mechanism of this synergism remains unclear at present, but residual viable cells that survived after the treatment with docetaxel permit the replication of Telomelysin, leading to effective cell death. Telomerase-dependent virotherapy has also been shown to overcome tumor resistance against chemotherapy in hepatocellular carcinoma.[112]

HDAC inhibitors increase *Coxsackie's-adenovirus receptor* (*CAR*) gene expression in various cancer cell lines.[113] In addition, they are known to increase viral and transgene expression following adenovirus infection.[113] In fact, FR901228, a potent HDAC inhibitor, activated CAR levels on target tumor cells, increasing the amounts of Telomelysin replication, leading to synergistic antitumor effects.[114] Selection of the partner chemotherapeutic agents appears to be an important factor that affects and determines the efficacy of telomerase-dependent oncolytic virotherapy.

Clinical trial of Telomelysin. A phase I clinical trial of Telomelysin as monotherapy has been performed in the United States. The proposed protocol 'A phase I dose-escalation study of intratumoral injection with telomerase-specific replication-competent oncolytic adenovirus, Telomelysin (OBP-301) for various solid tumors', sponsored by Oncolys BioPharma, is an open-label, phase I, three-cohort dose-escalation study. The trial commenced following the approval of the US Food and Drug Administration (FDA) in October 2006. The study is still underway and we plan to assess the safety, tolerability, and feasibility of intratumoral injection of the agent in patients with advanced cancer. We will also analyze the humoral immune response to Telomelysin, and take tissue biopsies to evaluate the pharmacokinetics and pharmacodynamics of Telomelysin in the injected tumor. The therapeutic response will be assessed by measuring changes in tumor dimensions, comparative analysis of tumor biopsies, and cytokine and/or viral measurements. Patients selected for this trial have histologically or cytologically proven nonresectable solid tumors and have failed to respond to conventional therapies such as primary external beam radiation or systemic chemotherapy. All patients have a disease that is measurable and accessible to direct injection of Telomelysin. The doses of Telomelysin will be escalated from low to high virus particles in 1-log increments. Patients will be treated with a single intratumoral injection of Telomelysin and then monitored for 1 month.

hTERT promoter for cancer diagnostics

A novel approach has been developed to visualize cancer cells using cancer-specific replication-competent adenovirus expressing the green fluorescent protein (GFP). Telomelysin was modified to contain the *GFP* gene driven by the cytomegalovirus (*CMV*) promoter in the E3-deleted region[115] (Fig. 2). The resultant adenovirus was termed TelomeScan or OBP-401. TelomeScan replicated 5–6 orders of magnitude by 3 days after infection in human cancer cell lines and coordinately induced GFP expression. In contrast, it replicated only 2 orders of magnitude in normal human fibroblasts without significant GFP expression. When TelomeScan was directly injected to subcutaneous xenografts of human cancer cells, the xenografts exhibited GFP signals over their entire area and were easily visualized, indicating that TelomeScan had replicated and spread throughout the tumors (Fig. 4a).

Adenoviral spread and subsequent replication at distal sites may also be useful to visualize the metastatic foci of cancers. Theoretically, replicated TelomeScan can pass through the lymphatic pathway from the primary tumors to the regional or sentinel lymph nodes and can replicate in metastatic foci. To this end, *in vivo* experiments were performed using colorectal tumor models which were orthotopically implanted into the rectum in mice.[116] This mouse model shows para-arotic lymph node metastasis after implantation, which was histologically confirmed. Some para-arotic lymph nodes exhibited GFP signals 24 h after intratumoral injection of TelomeScan into the primary site. Lymph nodes with GFP signals were dissected, followed by histological examination, and were found to have metastatic foci of the tumor cells, while those without GFP signals had no metastatic foci (Fig. 4b). The sensitivity and specificity of this imaging technique to detect metastatic foci are 92.3% and 86.6%, respectively.

This *in vivo* imaging model may be useful during surgical lymphadenectomy. After injecting TelomeScan into the primary tumor, the surgeon can visualize metastatic lymph nodes with GFP fluorescence by illuminating the abdominal cavity with a Xenon lamp. Of course, this diagnostic modality may also be applied as therapeutic modality. We confirmed that TelomeScan has lesser but still sufficient cytotoxic effects compared with Telomelysin (data not shown). Therefore, injected TelomeScan that spreads to the regional lymph nodes or other metastatic foci may have the ability to eradicate any remaining tumor cells that the surgeon fails to completely remove.

Finally, we are currently using TelomeScan as a tool to visualize cancer cells in cytological samples. Once exfoliated cells obtained from certain tissues are infected, the TelomeScan can replicate preferentially in *hTERT*-promoter-positive cancer cells and exhibit GFP signals that can easily been detected by fluorescent microscopy (Fig. 4c) (Maida *et al.*, manuscript in preparation).

Conclusion and perspectives

In the past decade, a number of factors that regulate *hTERT* transcription have been identified. However, no single factor accounts for the cancer-specific expression of *hTERT*. It is obvious that multiple factors are involved in its regulation, probably in combination, and chromatin remodeling appears to play a critical role. It is of particular interest that active chromatin marks present around the transcription start site of the *hTERT* promoter are tightly associated with unmethlated DNA in hTERT-positive cells, suggesting a mechanism that is consistent with the usual dynamics of gene regulation via DNA methylation. DNA methylation and modification of nucleosome histones such as acethylation and methylation are functionally linked and cooperate to regulate chromatin structure and gene expression. Emerging evidence suggests that some of the histone methyltransferases directly target the *hTERT* promoter. Studies of *hTERT* promoter regulation will be developed in relation to chromatin remodeling factors.

Clinical application of *hTERT* promoter as a driving promoter in oncolytic adenovirus has been realized in the past 5 years. Although several oncolytic adenoviruses have been developed, Telomelysin is the first hTERT-dependent oncolytic adnovirus to be used in a clinical trial. Several barriers appear to limit the efficacy of Telomelysin, probably including some tumor types being refractory to infection with Telomelysin due to low CAR expression, as well as the adverse effects on normal hTERT-positive cells. Revised Telomelysin, termed Telomelysin-RGD or OBP-405, has been developed, in which the virus fiber was modified to contain RGD (Arg-Gly-Asp) peptide, which binds with high affinity to integrins on the cell surface, leading to increased infectivity. We should consider the fact that some

doi: 10.1111/j.1349-7006.2008.00878.x

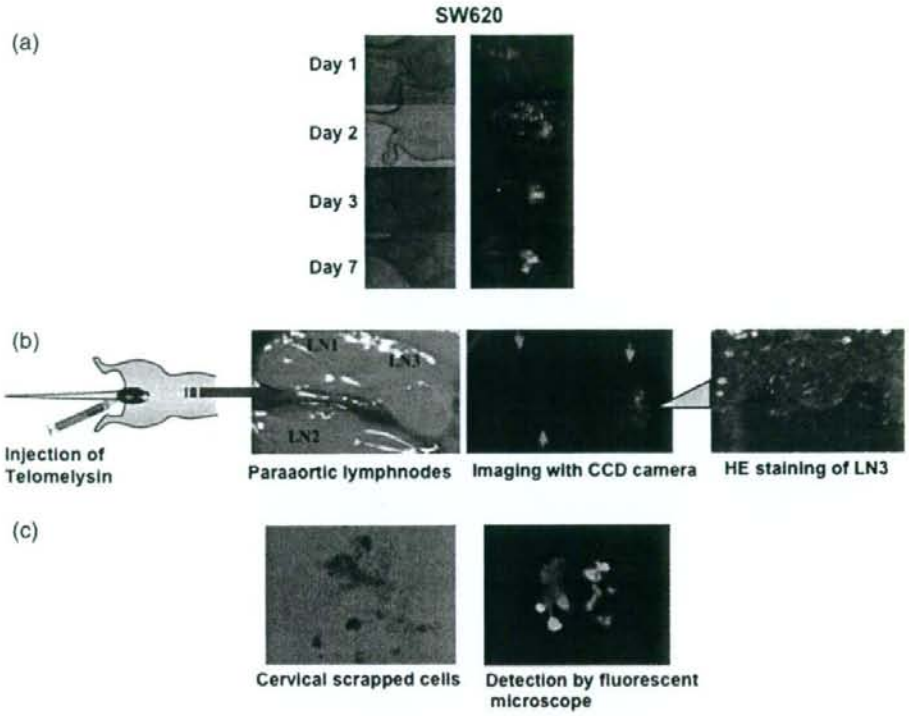

Fig. 4. Diagnostic utility of TelomeScan. (a) Visualization of tumor by the injection of TelomeScan. Subcutaneous tumor xenografts by colon cancer (SW620) were directly injected by TelomeScan at concentrations of 8×10^6 PFU. The green fluroescent protein (GFP) fluorescence intensity was monitored for seven consecutive days under the cooled charged-coupled device (CCD) imaging system. Left panels, macroscopic appearance of subcutaneous tumors; right panels, fluorescence detection. A modified version of this figure appeared in our original article.[114] (b) Selective visualization of lymph node metastasis by TelomeScan in orthotopic xenografts model. The rectums of mice were implanted with mouse rectal cancer HT29 cells. TelomeScan was directly injected into implanted tumor at a concentration of 1×10^8 PFU. At 5 days after the injection, mice were assessed for lymph node metastasis by laparotomy. Three swelled para-aortic lymph nodes were identified (LN1, LN2, and LN3). Internal imaging with the optical CCD camera showed one of the three nodes with GFP fluorescence (LN3), while the other lymph nodes (LN1, LN2) did not show (arrowheads indicate the position of swelled lymph nodes). Hematoxylin–eosin staining of lymph node sections revealed the apparent metastasis in LN3, while no metastatic sites were identified in LN1 or LN2 (data not shown), indicating that GFP fluorescence by the replication of TelomeScan is a potential biomaker of lymph node metastasis. A modified version of this figure appeared in our original article.[115] (c) Application of TelomeScan to visualization of cervical cancer cells in cytological samples. Uterine cervical scraping cells from patients with cervical cancer were incubated with TelomeScan at 10 MOI for 24 h, and then observed under light microscopy (left panel) or fluorescent microscopy (right panel). Clusters with cellular atypia exhibit GFP fluorescence.

normal cells, including some tissue stem cells, express relatively high levels of telomerase,[35,36,117] raising questions regarding the safety of Telomelysin. Although we have to wait for the final report of the clinical trial, no significant adverse effects on normal tissues have been reported so far, even in hematopoietic cells, which may be highly susceptible to Telomelysin due to the presence of telomerase-positive stem cells.[35] How can we explain such favorable phenomena? One possible explanation is that the hTERT promoter activity itself appears to be relatively lower in telomerase-positive normal cells than in hTERT-positive cancer cells, which limits its replication in normal cells and may largely contribute to the safety of this virus. Alternatively, Telomelysin may have lower capacity for infecting to hematopoietic stem cells possibly due to low CAR expression.[118]

Key to success of hTERT-dependent oncolytic virothrapy as a novel agent for cancer is a means of combining it with conventional therapies such as chemotherapy, radiotherapy, immunotherapy, surgery, or recently established molecular target therapies. The best combination and the timing of Telomelysin treatment (neoadjuvant, concurrent or adjuvant setting) should be investigated extensively in each tumor type.

Finally, diagnostic utility of hTERT-dependent oncolytic adenovirus for cancer may attract considerable attention in the near future. We began to apply this technology to cytological screening of cervical cancer and it should be extended to other tumor types for which cytological screening is important in early diagnosis. Intraoperative monitoring and detection of TelomeScan signals in the metastatic lymph nodes may provide revolutionary change in diagnostic modality during surgery. This novel technology will affect and contribute to the minimum operatin procedure for cancers.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan; and grants from the Ministry of Health, and Welfare of Japan; and Megumi Medical Foundation at Kanazawa University, Japan. We greatly appreciate all members of the molecular pathology group in the Department of Obstetrics and Gynecology, Kanazawa University, for their devoted contribution to work on telomerase in human cancer. We also thank Mr Yasuo Urata, president of Oncolys BioPharma, Tokyo, Japan, for the collaborative setting of the clinical trial for Telomelysin.

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doi: 10.1111/j.1349-7006.2008.00878.x

Oncogene (2008) 27, 2375–2381

www.nature.com/onc

ORIGINAL ARTICLE

Virus-mediated oncolysis induces danger signal and stimulates cytotoxic T-lymphocyte activity via proteasome activator upregulation

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Dendritic cells (DCs) are the most potent antigen-presenting cells and acquire cellular antigens and danger signals from dying cells to initiate antitumor immune responses via direct cell-to-cell interaction and cytokine production. The optimal forms of tumor cell death for priming DCs for the release of danger signals are not fully understood. OBP-301 (Telomelysin) is a telomerase-specific replication-competent adenovirus that induces selective E1 expression and exclusively kills human cancer cells. Here, we show that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human tumor cells, which in turn stimulated DCs to produce interferon-γ (IFN-γ) and interleukin 12 (IL-12). Subsequently, IFN-γ release upregulated the endogenous expression of the proteasome activator PA28 in tumor cells and resulted in the induction of cytotoxic T-lymphocytes. Our data suggest that virus-mediated oncolysis might be the effective stimulus for immature DCs to induce specific activity against human cancer cells.
Oncogene (2008) **27,** 2375–2381; doi:10.1038/sj.onc.1210884; published online 5 November 2007

Keywords: adenovirus; telomerase; dendritic cell; uric acid; danger signal

Introduction

Dendritic cells (DCs) are the most important professional antigen-presenting cells and play a critical role in the induction of primary immune responses against tumor-associated antigens. Mature DCs express high levels of major histocompatibility complex (MHC) class I, II and co-stimulatory molecules such as CD80 and CD86, and secrete T-helper type-1 (Th1) cytokines such as interleukin (IL)-12 and interferon (IFN)-γ. DCs acquire endogenous maturation stimuli from dying cells as a danger signal when they capture cellular antigens. Lack of danger signals delays maturation of DCs and causes active suppression of DCs stimulatory capacity, leading to the induction of T-cell tolerance (Steinman *et al.*, 2000). Shi *et al.* (2003) have previously identified uric acid as a novel endogenous warning molecule capable of alerting the immune system within cell lysates. The uric acid activates DCs following relocation from the inside to the outside of injured cells and converts immunity from non-protective to protective. In fact, it has been reported that uric acid levels are elevated in tumors undergoing immune rejection and that the inhibition of uric acid production delays tumor regression (Hu *et al.*, 2004).

Viruses have evolved to infect, replicate in and kill human cells through diverse mechanisms such as direct cell death machinery and fairly brisk immune responses. We reported previously that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES), induced selective E1 expression and efficiently killed human cancer cells, but not normal human fibroblasts (Kawashima *et al.*, 2004; Umeoka *et al.*, 2004; Taki *et al.*, 2005; Watanabe *et al.*, 2006). Although the precise molecular mechanism of OBP-301-induced cell death is still unclear, the process of oncolysis is morphologically distinct from apoptosis and necrosis. These findings led us to examine whether tumor cells killed by OBP-301 infection could stimulate DCs, thus enhancing the immune response.

In the present study, we compared three types of tumor preparations as a source of cell-derived antigen for the priming of DCs: virus-induced oncolysis, chemotherapeutic drug-induced apoptosis and necrosis by freeze/thaw. We also explored the cytokine signature and activating property of these cells for antitumor immune response against human cancer cells.

Results

We first examined whether OBP-301 infection affects the viability of human cancer cells using the XTT assay.

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Received 30 July 2007; revised 14 September 2007; accepted 17 September 2007; published online 5 November 2007