

telomerase activity. We also found that the number of viral genomes could be measured in genomic DNA purified from tissues of mice *in vivo* after injection of TRAD into the xenografts (Fig. 5). Although viral DNA could be detected even in normal tissues 70 days after intratumoral injection of TRAD, the absence of infectious virus as assessed by the plaque assay suggests that there are only DNA fragments in tissues. Our preliminary experiments have demonstrated that DNA could be isolated from tumors as small as 5 mm in diameter (data not shown). Therefore, the real-time PCR method with E1A and IRES primers permits rapid and quantitative detection of TRAD DNA in clinical samples.

We have shown recently the antiviral activity of cidofovir against TRAD *in vitro*. Cidofovir is an acyclic nucleoside phosphonate with potent broad-spectrum anti-DNA viral activity and has been approved for the treatment of many types of viruses, including cytomegalovirus and adenovirus.<sup>(13)</sup> Although viremia after TRAD administration is extremely rare because of the anti-adenovirus antibodies expected to be present in most patients, a

real-time PCR-based pharmacokinetic assay can allow the early detection of disseminated virus, and thus its use could provide an indication for commencement of cidofovir treatment in clinical trials.

In summary, we have established a fast, reliable, and sensitive assay to assess the biological activity of TRAD *in vitro* and to detect the viral genome in the plasma as well as tissues *in vivo*. A phase I clinical trial of TRAD targeting advanced solid tumors is currently underway in the USA following the approval of the Food and Drug Administration. Such an assay has been used in this ongoing trial and the data will be analyzed in the near future for the assessment of the safety, efficacy, and bio-distribution of TRAD.

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# Telomerase-Specific Oncolytic Virotherapy for Human Cancer with the hTERT Promoter

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**Abstract:** Replication-selective tumor-specific viruses present a novel approach for treatment of neoplastic disease. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor. For targeting cancer cells, there is a need for tissue- or cell-specific promoters that can express in diverse tumor types and are silent in normal cells. Recent advances in molecular biology have fostered remarkable insights into the molecular basis of neoplasm. Telomerase activation is considered to be a critical step in carcinogenesis and its activity correlates closely with human telomerase reverse transcriptase (hTERT) expression. Since only tumor cells that express telomerase activity would activate this promoter, the hTERT proximal promoter allows for preferential expression of viral genes in tumor cells, leading to selective viral replication. We constructed an attenuated adenovirus 5 vector (Telomelysin, OBP-301), in which the hTERT promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site (IRES). Telomelysin replicated efficiently and induced marked cell killing in a panel of human cancer cell lines, whereas replication as well as cytotoxicity was highly attenuated in normal human cells lacking telomerase activity. Thus, the hTERT promoter confers competence for selective replication of Telomelysin in human cancer cells, an outcome that has important implications for the treatment of human cancers. This article reviews recent findings in this rapidly evolving field: cancer therapeutic and cancer diagnostic approaches using the hTERT promoter.

**Keywords:** Telomerase, hTERT, adenovirus, GFP, imaging.

## INTRODUCTION

Human chromosomal end structures, named telomeres, serve as protective caps and consist of short tandemly repeated TTAGGG sequence [1, 2]. Telomere attrition contributes to genomic instability and may thereby promote the development of malignant cell transformation [3]. A fundamental difference in the behavior of normal versus tumor cells is that normal cells divide for a limited number of times, while tumor cells have the ability to proliferate indefinitely [4-6]. Telomere shortening sets a physical limit to the potential number of cell divisions and serves as a mitotic clock defining the lifespan of somatic cells [7]. One mechanism to escape this limitation is the activation or upregulation of telomerase. As telomerase can reset the mitotic clock, it has been linked to the processes of tumorigenesis and aging. Telomerase is a ribonucleoprotein complex responsible for adding TTAGGG repeats onto the 3' ends of chromosomes [8-10]. Many studies have demonstrated that the majority of malignant tumors express telomerase activity, a feature that accounts for their proliferative capacity [11-13], whereas telomerase is strongly repressed in most normal somatic tissues [14]. Therefore, telomerase has attracted considerable attention as a plausible target for cancer diagnosis and therapy [15].

The human telomerase complex is composed of three components: the RNA subunit (known as hTR, hTER, or hTERC) [16], the telomerase-associated protein (hTEP1) [17], and the catalytic subunit (hTERT, human telomerase reverse transcriptase) [18, 19]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [20] and, therefore,

represent the minimal catalytic core of telomerase in humans [21]. However, while hTR is widely expressed in embryonic and somatic tissue, hTERT is tightly regulated and is not detectable in most somatic cells. The cloning of the promoter region of hTERT in 1999 [22-25] facilitated the development of targeted cancer gene therapy approaches that can specifically and markedly augment transgene expression in tumor with its specificity. Telomerase-specific expression of cytotoxic or proapoptotic genes such as the diphtheria toxin A-chain, FADD, caspases, Bax, and PUMA by the hTERT promoter has been successfully achieved and reported in various gene transfer systems (e.g., plasmid and adenovirus) [26-31]. Although adenovirus-mediated Bax gene expression via the hTERT promoter elicits a therapeutic effect on tumor cells and could prevent the toxic effects on normal cells [30], the viral spread might be less than ideal after intratumoral administration.

Replication-defective, E1-deleted adenoviral vectors facilitate the efficient delivery of a variety of transgenes to target tissues and have demonstrated clear therapeutic benefits and safety in a variety of clinical studies [32-34]; a significant obstacle, however, is the limited distribution of the vectors within the tumor mass even after direct intratumoral administration. To confer specificity of infection and increase viral spread to neighboring tumor cells, the notion of using replication-competent adenoviruses has become a reality [35-37]. The fact that activation of hTERT gene expression is one of the key events during tumorigenesis [38, 39] enables the hTERT promoter to take place in the tumor-specific transcriptional control of genes essential for viral replication. We hypothesized that an adenovirus containing the hTERT promoter-driven E1 genes could be used to target a variety of tumor cells and kill them efficiently by viral replication. Moreover, this virus can be useful for cancer diagnostics, especially for detection of minute metastases *in vivo*, since more than 85% of human cancers display telomerase activity [12].

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## TELOMERASE AND CANCER

### Telomerase Activation in Human Cancer

Cancer is characterized by unregulated proliferation of a certain cell population, which eventually affects normal cellular function in the human body [4-6]. To selectively target cancer cells, it is essential to identify the crucial molecular determinants involved in tumor progression. Cellular immortality is a critical step in tumorigenesis and, therefore, the molecular mechanism of the unlimited replicative capacity of tumor cells may provide universal and effective means for treating human cancer [15].

Telomeres are situated at the ends of linear chromosomes and protect them from degradation and end-to-end fusions [2]. Tumor cells can maintain telomere length predominantly due to the enzyme telomerase [8-10]. Telomerase activity is detected in about 85% of malignant tumors [12], whereas in most normal somatic tissues telomerase is absent [14]. Although weak telomerase activity is detected in peripheral blood leukocytes and in certain stem cell population [40, 41], the majority of malignant tumors express high levels of telomerase activity [11-13]. There is also a gradient increase in telomerase activity between early and late stage tumors. The strong association between telomerase activity and malignant tissue suggests that telomerase can be an essential target for the diagnosis and treatment of cancer.

The transcriptional upregulation of hTERT, a catalytic subunit of telomerase, represents the rate-limiting step in telomerase expression [18, 19], although other pathways involved in the control telomerase activity such as differential splicing of the hTERT transcript and posttranscriptional modification of the hTERT protein may exist [42]. Thus, the hTERT promoter region can be used as a fine-tuning molecular switch that works exclusively in tumor cells.

### Regulation of hTERT Transcription

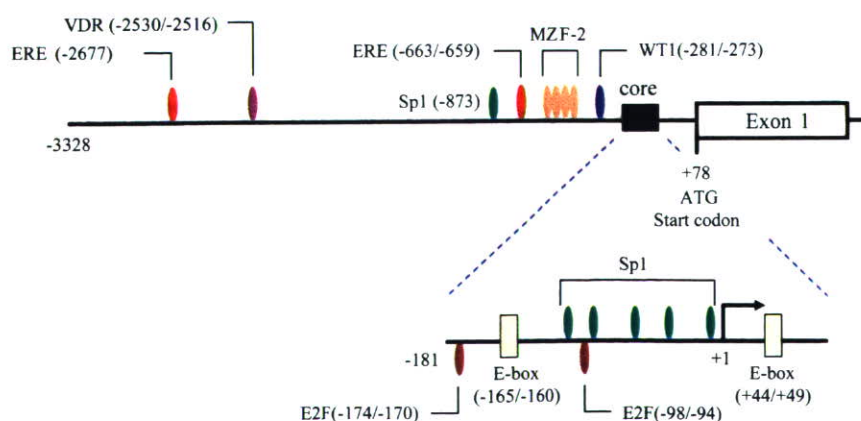
Recent studies have provided mechanistic insight into how the hTERT promoter can be stimulated or suppressed by oncogenic activation as well as inactivation of tumor suppressors. Various laboratories have identified transcription factors that are involved in upregulation or downregulation of hTERT transcriptional activity (Fig. 1). These reports proposed a variety of potential mechanisms of the transcriptional control

of hTERT, which may help us design telomerase- or hTERT-based cancer therapies.

The hTERT promoter contains two E-boxes (CACGTG) that are binding sites for the Myc/Max/Mad network of transcriptional factors [22, 24, 43, 44]. The oncoprotein c-Myc forms a complex with the Max protein that binds as a heterodimer to activate hTERT transcription. In contrast, heterodimers with Mad1 and Max proteins result in repression of hTERT expression [45, 46]. The relative levels of c-Myc and Mad1 correlate directly with activation and repression of hTERT expression. The transcriptional factor Sp1 has been reported to cooperate with c-Myc to induce the hTERT promoter, depending on cell type, suggesting a reliance on Sp1 for full activity of c-Myc [47]. Other transcriptional factors such as ETS proteins and viral proteins also contribute to hTERT upregulation. Since epidermal growth factor (EGF) receptor and its homolog, the HER2/Neu proto-oncoprotein, stimulate phosphorylation of MAP kinases [48], which in turn activate ETS1/ETS2 [49], stimulation by EGF can lead to hTERT upregulation. The human papilloma virus (HPV) type 16 E6 protein can also associate with c-Myc and thereby activate the hTERT promoter [50-52].

In addition to Mad1, several dominant repressors that mediate hTERT downregulation have been identified. For example, the Wilm's tumor suppressor 1 (WT1) and myeloid-specific zinc finger protein 2 (MZF-2) interact with the hTERT promoter, to suppress hTERT transcription [53, 54]. Based on the preferential expression of WT1 in kidney, gonads, and spleen and of that of MZF-2 in myeloid cells, WT1 and MZF-2-mediated repression of hTERT seems tissue-specific. Other transcriptional factors, E2F-1, E2F-2, and E2F-3, also repress hTERT transcription by binding to the hTERT promoter [55, 56].

The hTERT transcription is also regulated by nuclear hormones as well as drugs that involve gene expression. Estrogen induces an increase in hTERT mRNA levels through the estrogen receptor (ER), which interacts with two estrogen response elements (EREs) in the hTERT promoter [57, 58]. Progesterone and androgen also stimulate telomerase activity through hTERT expression, although this response is likely to be indirect [59]. Furthermore, histone deacetylase (HDAC) inhibitors activate the transcription of certain genes by altering the acetylation status of nucleosomal histones. It has been reported that treatment with HDAC inhibitor, trichostatin A (TSA), could induce significant activation of hTERT mRNA



**Fig. (1).** Scheme of the proximal promoter of hTERT. Putative protein binding sites for various transcription factors are indicated.

expression and telomerase activity in normal cells through the TSA-responsive element localized in the hTERT proximal promoter [60]. In contrast, nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, and cyclooxygenase (COX)-2 inhibitor have been recently shown to inhibit telomerase activity at the hTERT transcriptional level in colon cancer cells [61]. The *cis*-response elements to NSAIDs have been identified in the hTERT promoter region. Furthermore, some nuclear hormone receptors including vitamin D receptor and retinoic acid receptor can repress hTERT expression [62, 63].

These observations gained from the study of hTERT transcriptional regulation suggest that hTERT activity in cancer cells can be modified by exogenous stimuli such as hormones, drugs, and genes, which may enhance the anti-tumor effects of hTERT-specific cancer therapies as combined modalities.

## hTERT PROMOTER FOR CANCER THERAPEUTICS

### Construction of Telomelysin

The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for treatment of cancer. One approach to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as E1A or E4. For example, the heterologous promoters from the prostate-specific antigen (PSA) [64], MUC1 [65], osteocalcin [66], L-plastin [67], midkine [68], and E2F-1 [69] genes have been used to drive E1A expression. These vectors replicate preferentially in tumor cells that express each targeted tumor marker; their therapeutic window, however, is relatively narrow because only part of the tumor is positive for each tumor marker. As described above, telomerase, especially its catalytic subunit hTERT, is expressed in the majority of human cancers and the hTERT promoter is preferentially activated in human cancer cells [12]. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. Indeed, it has been reported previously that the transcriptional control of E1A expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells [70-72].

The adenovirus E1B gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promote the cytoplasmic accumulation of late viral mRNA, leading to a shut down of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the E1A gene including hTERT-specific oncolytic adenoviruses, the E1B gene is driven by the endogenous adenovirus E1B promoter. However, Li *et al.* have demonstrated that transcriptional control of both E1A and E1B genes by the  $\alpha$ -fetoprotein (AFP) promoter with the use of IRES significantly improved the specificity and the therapeutic index in hepatocellular carcinoma cells [73]. Therefore, we have developed Telomelysin (OBP-301), in which the tumor-specific hTERT promoter regulates both the E1A and E1B genes (Fig. 2). Telomelysin controls the viral replication more stringently, thereby providing profound therapeutic effects in tumor cells as well as the attenuated toxicity in normal tissues [74].

The construction of Telomelysin was carried out as follows. An 897-bp fragment of the E1A gene and a 1822-bp fragment of

the E1B gene were amplified by PCR from cellular RNA and genomic DNA of 293 cells, respectively. The amplified products were subcloned into the pTA plasmid. Following confirmation by DNA nucleotide sequencing, the E1A (911 bp) and E1B (1836 bp) genes were cloned into the pIRES vector (pE1A-IRES-E1B). A 455-bp fragment of the hTERT promoter, which contains a 378-bp region upstream of the transcription start site, was ligated into the pE1A-IRES-E1B (phTERT-E1A-IRES-E1B). The 3828-bp fragment was digested from the phTERT-E1A-IRES-E1B and then cloned into pShuttle after deletion of the cytomegalovirus (CMV) promoter. The resultant shuttle vector was applied to the Adeno-X Expression System (Clontech Laboratories, Palo Alto, CA). Recombinant adenovirus was isolated from a single plaque and expanded in 293A cells. The resultant virus was termed Telomelysin.

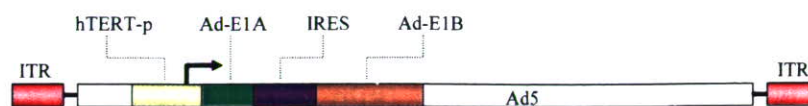
The 181-bp fragment upstream of the transcription start site is considered the core functional promoter that is essential for transcriptional activation of hTERT in tumor cells. Takakura *et al.* reported by analysis of 5'-truncations of the promoter that hTERT transcriptional activity decreased with deletion of sequences between -776 and -1375 and increased with the deletion of sequences between -378 and -776, indicating that *cis*-acting and silencer elements, respectively, exist in these regions [22]. They also demonstrated that the 378-bp fragment that we used for Telomelysin could exhibit high transcriptional activity similar to that of the 181-bp core promoter region.

### Functional Analysis of Telomelysin

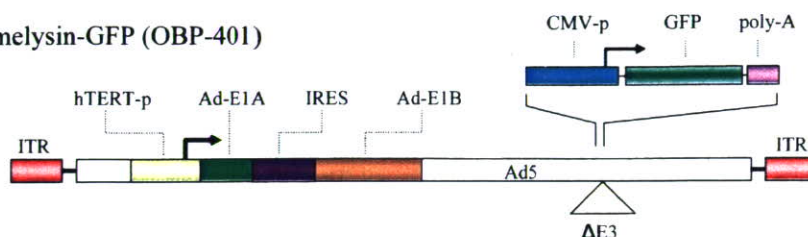
Methods used for measuring viral replication of Telomelysin include standard plaque assay using 293 cells as well as quantitative real-time PCR analysis targeting for the viral E1A or IRES sequence [74, 75], both of which present similar replication patterns of Telomelysin in human cancer cells. Telomelysin induced selective E1A and E1B expression in cancer cells, which resulted in viral replication at 5-6 logs by 3 days after infection; Telomelysin replication, however, was attenuated up to 2 logs in cultured normal cells [74, 75]. Although the transduction efficiency of adenovirus is less efficient in normal cells compared with tumor cells, the observation that wild-type adenovirus infection killed normal cultured cells more effectively suggests that the attenuated cytotoxicity of Telomelysin in normal cells is due to tumor-specific replication, but not due to the low transduction. These data indicate that selective replication of Telomelysin is both therapeutically beneficial and safe. The relative E1A DNA levels determined by quantitative real-time PCR assay after Telomelysin infection correlated with hTERT mRNA expression levels in several human cancer cell lines, suggesting that Telomelysin viral yields are closely associated with the hTERT transcriptional activity in human cancer.

The majority of human cancer cells acquire immortality and unregulated proliferation by expression of the hTERT [12] and, therefore theoretically, hTERT-specific Telomelysin can possess a broad-spectrum antineoplastic activity against a variety of human tumors. *In vitro* cytotoxicity assays demonstrated that Telomelysin could efficiently kill various types of human cancer cell lines including head and neck cancer, lung cancer, esophageal cancer, gastric cancer, colorectal cancer, breast cancer, pancreatic cancer, hepatic cancer, prostate cancer, cervical cancer, melanoma, sarcoma, and mesothelioma in a dose-dependent manner. The dose of Telomelysin that causes

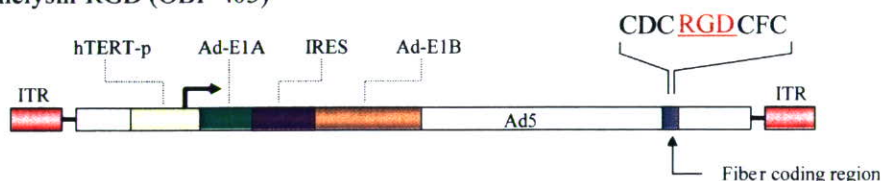
## Telomelysin (OBP-301)



## Telomelysin-GFP (OBP-401)



## Telomelysin-RGD (OBP-405)



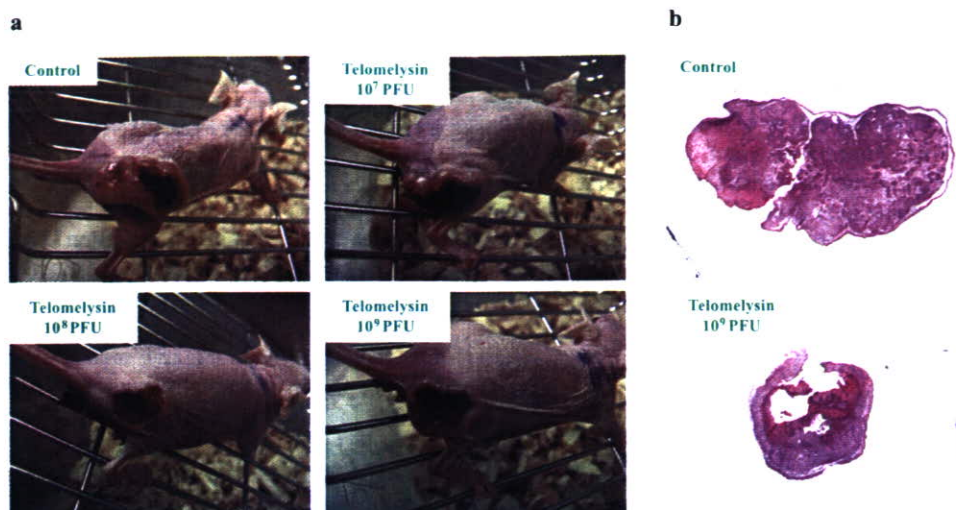
**Fig. (2).** Schematic DNA structures of telomerase-specific oncolytic viruses. Telomelysin (OBP-301), in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an IRES. Telomelysin-GFP (OBP-401) is a telomerase-specific replication-competent adenovirus variant, in which GFP gene is inserted under CMV promoter into E3 region for monitoring viral replication. Telomelysin-RGD (OBP-405) has mutant fiber containing the RGD peptide, CDCRGDCFC, in the HI loop of the fiber knob.

about 50% reduction in cell viability in monolayer cultures (defined as  $ID_{50}$ ) was less than 20 multiplicity of infections (MOIs) in almost all tumor cell lines examined in our study. These data clearly demonstrate that Telomelysin exhibits desirable features for use as an oncolytic therapeutic agent, as the proportion of cancers potentially treatable by Telomelysin is extremely high.

The *in vivo* antitumor effect of Telomelysin was also investigated by using athymic mice carrying xenografts. Intratumoral injection of Telomelysin into human tumor xenografts resulted in a significant inhibition of tumor growth and enhancement of survival [74, 75]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose Telomelysin, indicating that Telomelysin induced intratumoral necrosis of tumor cells due to direct lysis by virus replication *in vivo* (Fig. 3). For effective treatment of distant metastatic tumors, intravenously infused chemotherapeutic drugs will need to distribute in sufficient quantities into the tumor sites; oncolytic viruses, however, could still replicate in the tumor, cause oncolysis, and then release virus particles that could reach the distant metastatic lesions. Therefore, intratumoral administration that causes the release of newly formed virus from infected tumor cells might be theoretically suitable for oncolytic virus rather than systemic administration. Indeed, it was confirmed that, following intratumoral injection, Telomelysin replicated within tumors, spread into the bloodstream, and then replicated in distant tumor sites [74, 75]. The biodistribution of Telomelysin as assessed by PCR amplification targeting for the viral E1A

provides evidence that viral replication is highly specific for tumors despite its presence in the circulation. No significant elevation of liver enzymes was observed in mice intratumorally injected with Telomelysin. In addition, histopathological analysis of liver sections demonstrated absence of apoptotic hepatocytes and other histological signs of hepatocellular damage [75].

Chemotherapeutic drugs kill tumor cells mainly by inducing apoptosis, which is characterized by chromosome condensation and nuclear shrinkage and fragmentation; nuclear morphology of cells infected with Telomelysin, however, was distinct from apoptosis. Apoptosis in mammalian cells is mediated by a family of cysteine proteases known as caspases, which are the executioners of apoptosis and essential for the disassembly of the cell. No changes in procaspase-3 levels and no expression of cleaved form of caspase-3 in cells infected with Telomelysin were noted. Moreover, flow cytometric analysis demonstrated that Telomelysin infection had no effect on cell cycle distribution [76, 77]. Recently, Ito *et al.* have reported that hTERT-specific oncolytic adenovirus causes autophagic cell death, which is a type of programmed cell death that is an alternative to apoptosis, in malignant glioma cells via inhibition of the mTOR signal [78]. Although their data clearly indicate that autophagy may be one of the cell death machinery induced by oncolytic adenoviruses, our preliminary studies using the green fluorescent protein (GFP) and microtubule-associated protein 1 light chain 3 (LC3) fusion plasmid (GFP-LC3) [79] demonstrated that Telomelysin did not induce GFP-LC3 dots, which represent pre-autophagosomes and



**Fig. (3).** Antitumor effect of intratumorally injected Telomelysin against established flank SW620 xenograft tumors in *nu/nu* mice. **(a)** Macroscopic appearance of tumors 15 days after treatment with various concentrations of Telomelysin. **(b)** Tumors were dissected 15 days after viral injection and paraffin sections were stained with hematoxylin and eosin. Massive tumor cell death at the central portions of the tumors where Telomelysin was injected was observed.

autophagosomes in human lung cancer cells. Thus, further investigation in other types of cancer cells will be required to determine the exact mechanisms of Telomelysin-triggered cell death.

#### Multi-Disciplinary Therapy with Telomelysin

The development of Telomelysin as a monotherapy is currently underway clinically based on the promising results of preclinical studies; multi-modal strategies to enhance antitumor efficacy *in vivo*, however, are essential for successful clinical outcome. In fact, most of the clinical trials for oncolytic viruses have been conducted in combination with chemotherapy or radiotherapy [80-83]. In a report of clinical trial of ONYX-015, no clinical benefit was noted in the majority of patients, despite the encouraging biological activity [84]. Tumor progression was rapid in most patients, even though substantial necrosis was noted in the tumors after treatment [85, 86]. Therefore, multi-disciplinary therapy composed of oncolytic virotherapy combined with low-dose chemotherapeutic agent is required to enhance the antitumor efficacy. Moreover, combination of two agents may allow the use of reduced dosage of each agent, and reduce the likelihood of adverse effects.

Infection with Telomelysin (GFP-expressing Telomelysin was used as an alternative to Telomelysin in some experiments) alone or followed by treatment with docetaxel (Taxotere), a chemotherapeutic agent, resulted in a profound *in vitro* cytotoxicity in various human cancer cell lines originating from different organs (lung, colon, esophagus, stomach, liver, and prostate), although the magnitude of antitumor effect varied among the cell types [77]. Other chemotherapeutic drugs such as vinorelbine (Navelbine) and SN38 (the potent active metabolite of irinotecan) combined with Telomelysin also inhibited the growth of human cancer cells [77]. Quantitative real-time PCR analysis demonstrated that docetaxel did not affect viral replication. For *in vivo* evaluation, mice xenografted with human lung tumor received intratumoral injection of Telomelysin and intraperitoneal administration of docetaxel.

Analysis of growth of implanted tumors showed a significant, therapeutic synergism, while Telomelysin alone and docetaxel alone showed modest inhibition of tumor growth [77]. The antitumor effect of the combination therapy was likely additive *in vitro*; there might be, however, some particular interactions between Telomelysin and docetaxel to produce a synergistic effect *in vivo*. It has been reported that metronomic chemotherapy, which refers to long-term administration of comparatively low doses of cytotoxic drugs at close, regular intervals, has an antiangiogenic basis [87]. Like our approach, the potent antiangiogenic capacity of drugs administered in a metronomic fashion finds favor in a number of *in vivo* preclinical studies; to prove this efficacy by *in vitro* experiments is, however, technically difficult. There are some possible explanations for the superior *in vivo* antitumor activity in our experiments. Systemically administered docetaxel may attack the vascular endothelial cells at the tumor site, which in turn can block the escape of locally injected Telomelysin into the blood circulation. Another possibility is that Telomelysin itself may inhibit the vascular supply by killing endothelial cells.

FR901228 (depsipeptide, FK228) is a novel anticancer agent isolated from the fermentation broth of *Chromobacterium violaceum*. FR901228 has been identified as a potent histone deacetylase (HDAC) inhibitor. Histone deacetylation is an important component of transcriptional control, and it has been shown that FR901228 can increase Cocksackie's-adenovirus receptor (CAR) gene expression in various cancer cell lines [88-91]. Moreover, FR901228 is known to increase viral and transgene expression following adenovirus infection [88]. Indeed, FR901228 treatment upregulated CAR levels on target tumor cells, which in turn increased the amount of cellular Telomelysin replication, thereby promoting a synergistic antitumor effect [76]. These data indicate that FR901228 may be an appropriate partner for Telomelysin because it does not affect the virus life cycle. Delineating specific virus/drug combinations that are tailored to be particularly effective in human cancer could potentially improve the already encouraging results seen in the field of oncolytic virotherapy.

## Clinical Application of Telomelysin

Preclinical models suggested that Telomelysin could selectively kill a variety of human cancer cells *in vitro* and *in vivo* via intracellular viral replication regulated by the hTERT transcriptional activity. Pharmacological and toxicological studies in mice and cotton rats demonstrated that none of the animals treated with Telomelysin showed signs of viral distress (e.g., ruffled fur, weight loss, lethargy, or agitation) or extensive histopathological changes in any organs at autopsy. These promising data led us to design a phase I clinical trial of Telomelysin as a monotherapy.

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, Inc. is an open-label, phase I, 3 cohort dose-escalation study. The Recombinant DNA Advisory Committee (RAC) at the National Institutes of Health (NIH) has already reviewed this protocol. The safety, tolerability, and feasibility of intratumoral injection of the agent will be assessed in patients with advanced cancer. The humoral immune response to Telomelysin will be analyzed also. Biopsies will be taken to evaluate the pharmacokinetics and pharmacodynamics of Telomelysin in the injected tumor. 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, non-resectable solid tumors and exhibited lack of response to conventional therapies such as primary external beam radiation or systemic chemotherapy. Patients have a disease that is measurable and accessible to direct injection of Telomelysin. Doses of Telomelysin will be escalated from low to high virus particles (VP) in one log increment. Patients will be treated with a single dose intratumoral injection of Telomelysin and then monitored for one month. The trial has been started upon approval of the US Food and Drug Administration (FDA) on November, 2006.

The data of pharmacokinetics and biodistribution of Telomelysin will be of interest. In the phase I trial of Advexin, a replication-deficient adenoviral vector that delivers normally functioning p53 tumor suppressor gene to cancer cells, the vector was present in tumor tissue as well as proximal lymph nodes, indicating regional spread of the vector via the lymphatic vessels [92]. Moreover, clinical trials of intratumoral and intravenous administration of CG7870, a replication-selective oncolytic adenovirus genetically engineered to replicate preferentially in prostate tissue, demonstrated a second peak of the virus genome in the plasma [93, 94], suggesting active viral replication and shedding into the bloodstream. Therefore, it is anticipated that intratumorally administered Telomelysin can spread into the lymphatic vessels as well as the blood circulation, and potentially kill metastatic tumor cells in regional lymph nodes and distant organ tissues. Theoretically, Telomelysin can replicate continuously in the injected tumors and releases virus particles unless all tumor cells are completely eliminated, indicating that a single intratumoral injection should be sufficient to induce antitumor effect. Our preclinical study, however, showed that multiple injections of Telomelysin resulted in a profound inhibition of tumor growth in xenograft models [74, 75, 77]. Thus, once the safety of a single administration is confirmed, the feasibility of

the multi-cycle treatment with Telomelysin will be assessed in human.

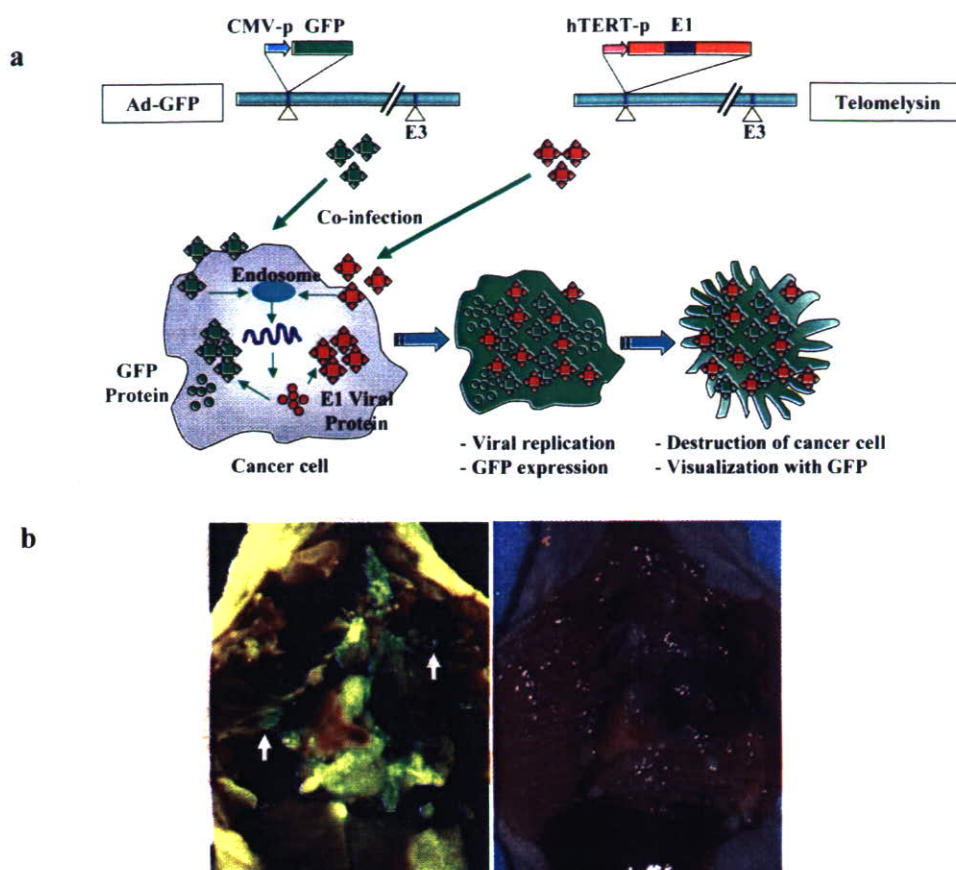
## hTERT PROMOTER FOR CANCER DIAGNOSTICS

### Imaging of Tumor Cells using Telomelysin-GFP

A variety of imaging technologies is being investigated as tools for cancer diagnosis, detection, and treatment monitoring. Improvements in methods of external imaging such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound techniques have increased the sensitivity for visualizing tumors and metastases in the body [95]; a limiting factor in structural and anatomical imaging, however, is the inability to specifically identify malignant tissues. Positron emission tomography (PET), with the glucose analogue  $^{18}\text{F}$ -2-deoxy-D-glucose (FDG), is the first molecular imaging technique that was widely applied for cancer imaging in clinical settings [96]. Although FDG-PET has high detection sensitivity, it has some limitations such as difficulty in distinguishing between proliferating tumor cells and inflammation and unsuitability for real-time detection of tumor tissues. Therefore, tumor-specific imaging would be of considerable value in treatment of human cancer by defining the location and area of tumors without microscopic analysis. In particular, if tumors too small for direct visual detection and therefore not detectable by direct inspection could be imaged *in situ*, surgeons could precisely excise tumors with appropriate surgical margins. This paradigm requires an appropriate "marker" that can facilitate visualization of physiological or molecular events that occur in tumor cells but not normal cells.

The green fluorescent protein (GFP), which was originally obtained from the jellyfish *Aequorea Victoria*, is an attractive molecular marker for imaging in live tissues because of the relatively non-invasive nature of fluorescent [97]. A new approach developed in our laboratories to specifically visualize human tumor cells involves the use of Telomelysin and a replication-deficient adenovirus expressing the GFP gene (Ad-GFP) (Fig. 4). Telomelysin infection could complement E1 gene functions and facilitate replication of E1-deleted Ad-GFP selectively in co-infected tumor cells [98]. When the human cancer cell lines were infected with Ad-GFP at low MOI, GFP expression could not be detected; in the presence of Telomelysin, however, Ad-GFP replicated in these tumor cells and showed strong green signals. By contrast, co-infection of Telomelysin and Ad-GFP did not show any fluorescence in normal cells such as fibroblasts and vascular endothelial cells because of the low levels of hTERT activity. This strategy was also applied successfully *in vivo*; intrathoracic administration of Telomelysin and Ad-GFP clearly labeled disseminated human lung tumor nodules in mice under the cooled charged-coupled device (CCD) camera (Fig. 4). These data indicate that locoregional injection of Telomelysin plus Ad-GFP in combination with the highly sensitive CCD imaging system might be a useful diagnostic tool for real-time visualization of macroscopically invisible tumor tissues.

The advantage of co-infection of an E1-deleted replication-deficient adenoviral vector and Telomelysin is that transgene expression can be amplified in target cells. Furthermore, many vectors previously constructed can be used to express genes of interest. However, the requirement for both viruses to infect the same cell for the amplified transgene expression is a significant limitation of this dual virus vector system. The degree of



**Fig. (4).** (a) Concept of selective visualization of tumor cells with Ad-GFP and Telomelysin. (b) Internal images of pleural dissemination visualized by intrathoracic injection of Ad-GFP and Telomelysin. Female BALB/c *nu/nu* mice received intrathoracic implant with A549 human lung tumor cells. Five days after Ad-GFP and Telomelysin injection into the thoracic space, mice were sacrificed, and their thoracic spaces were examined. Fluorescent detection of disseminated tumors. Arrows, disseminated tiny tumor.

transgene expression has been shown to vary depending on the copy numbers of the viruses that initially infected the cells. To label efficiently and uniformly target tumor cells with green fluorescence, we modified Telomelysin to contain the GFP gene driven by the cytomegalovirus (CMV) promoter in the E3 deleted region (Fig. 2). The resultant adenovirus was termed Telomelysin-GFP or OBP-401 [76, 77]. Similar to Telomelysin, Telomelysin-GFP replicated 5-6 logs by 3 days after infection in human cancer cell lines and coordinately induced GFP expression; Telomelysin-GFP replication, however, was attenuated up to 2 logs in normal human fibroblasts without GFP expression. Subcutaneous human tumor xenografts could be visualized after intratumoral injection of Telomelysin-GFP. Tumor sections entirely expressed GFP, suggesting *in vivo* viral replication and spread throughout the tumors.

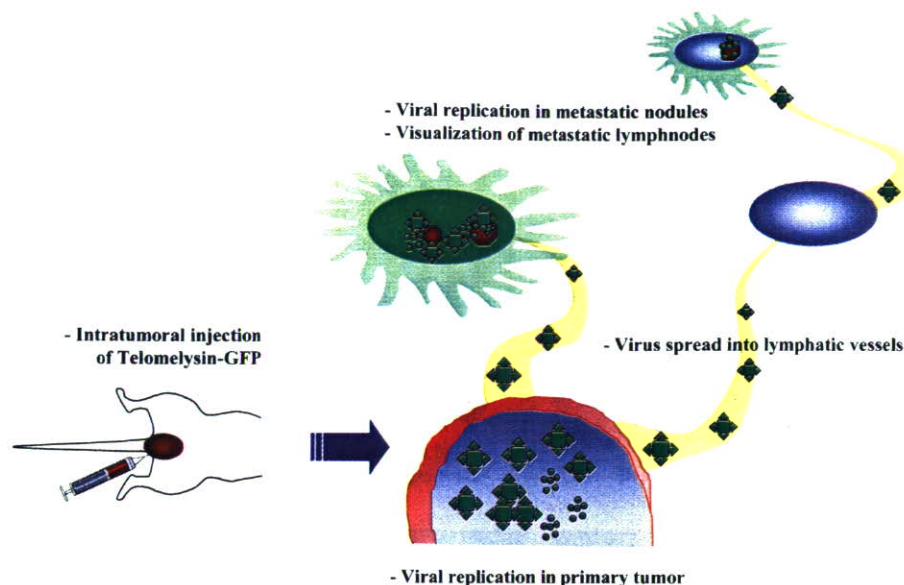
#### ***In vivo* Imaging of Metastatic Tumor Cells with Telomelysin-GFP**

Metastatic spread of tumor cells plays a major role in the morbidity and mortality of human cancer. Although there are few life-prolonging treatments for the majority of patients with distant sites of metastasis, early detection of occult metastasis and early therapeutic interventions may decrease the rate of metastatic spread and extend survival. Lymphatic invasion is one of the major routes for cancer metastasis, and adequate resection of locoregional lymph nodes is required for curative

treatment in patients with advanced malignancies. The risk of lymph node metastasis can be partially predicted by clinical data such as tumor stage, serum tumor marker level, and medical images; there are, however, no noninvasive approaches to accurately predict the presence of lymph node metastasis, in particular, microscopic metastasis. Although molecular analysis based on detection of genetic markers of cancer cells is clinically relevant in some patients, the procurement of sufficient tissue to confirm the diagnosis can be associated with significant morbidity and cost depending on the size and location of the lesion. Therefore, the utility of Telomelysin-GFP that can be used for real-time imaging of tumor tissues *in vivo* offers a practical, safe, and cost-effective alternative to the traditional, cumbersome procedures of histopathological examination.

Following intratumoral injection of Telomelysin-GFP into human colorectal tumors orthotopically implanted into the rectum in mice, para-aortic lymph node metastasis could be visualized at laparotomy under a CCD camera. Histopathological analysis confirmed the presence of metastatic adenocarcinoma cells in the lymph nodes with fluorescence emission, whereas GFP-negative lymph nodes contained no tumor cells. Of interest, metastatic lymph nodes were imaged in spots with GFP fluorescence, which was in agreement with histologically-confirmed micrometastasis. The sensitivity and specificity of this imaging technique are 92.3% and 86.6%,





**Fig. (5).** Concept of selective visualization of lymph node metastasis with Telomelysin-GFP.

respectively, which are sufficiently reliable to support the concept of this approach [99]. These data indicate that Telomelysin-GFP causes viral spread into the regional lymphatic area and selectively replicates in neoplastic lesions, resulting in GFP expression in metastatic lymph nodes (Fig. 5). This experiment mimics the clinical scenario where patients with gastrointestinal malignancies and lymph node metastasis undergo surgery, and the data suggest that the surgeon can identify metastatic lymph nodes by illuminating the abdominal cavity with a Xenon lamp.

Administration of Telomelysin-GFP offers an additional advantage in cancer therapy. Telomelysin-GFP, similar to Telomelysin, is an oncolytic virus, and selectively kills human tumor cells by viral replication; the process of cell death by Telomelysin-GFP, however, is relatively slow compared to apoptosis-inducing chemotherapeutic drugs, because the virus needs time for replication. Therefore, tumor cells infected with Telomelysin-GFP express GFP fluorescence, followed by loss of viability, allowing the timing of detection. Thus, Telomelysin-GFP can spread into the regional lymph nodes after intratumoral injection, express GFP signals in tumor cells by virus replication, and finally kill tumor cells even if the surgeon failed to remove all nodes containing micrometastasis.

## CONCLUSION AND PERSPECTIVES

There have been very impressive advances in our understanding of the molecular aspects of human cancer and in the development of technologies for genetic modification of viral genomes. Nevertheless, there are many remaining hurdles, ethical and technical that must be solved before virotherapy including virus-mediated gene therapy ever reach routine clinical application. The safety considerations in the virus manufacture and clinical protocols are among the most important issues to be studied. Another important issue is to find ways to selectively deliver viruses into a high percentage of malignant cells in an existing tumor mass. The use of tissue or cell-type specific promoters could perhaps achieve

specificity of virus-mediated antitumor effect. The hTERT promoter-based transcriptional targeting in adenoviral constructs is a powerful tool for cancer diagnosis and therapy. In particular, the hTERT-specific oncolytic adenovirus achieves a more strict targeting potential due to the amplified effect by viral replication, and is a promising therapeutic alternative to replication-deficient gene therapy vectors. Several independent studies that used different regions of hTERT promoter and different sites of adenoviral genome responsible for viral replication, have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effect in a variety of human tumor cells [70-72, 74]. Among these viral constructs, to the best of our knowledge, Telomelysin seems to be the first hTERT-dependent oncolytic adenovirus that has been used in a clinical trial based on preclinical pharmacological and toxicological studies.

Although Telomelysin showed a broad and profound antitumor effect in human cancer originating from various organs, one weakness of Telomelysin is that virus infection efficiency depends on CAR expression, which is not highly expressed on the cell surface of some types of human cancer cells. Thus, tumors that lost CAR expression may be refractory to infection with Telomelysin. Since modification of fiber protein is an attractive strategy for overcoming the limitations imposed by the CAR dependence of Telomelysin infection, we modified the fiber of Telomelysin to contain RGD (Arg-Gly-Asp) peptide, which binds with high affinity to integrins ( $\alpha v \beta 3$  and  $\alpha v \beta 5$ ) on the cell surface, on the HI loop of the fiber protein (Fig. 2). The resultant adenovirus, termed Telomelysin-RGD or OBP-405, mediated not only CAR-dependent virus entry but also CAR-independent, RGD-integrin-dependent virus entry [75]. Telomelysin-RGD had an apparent oncolytic effect on human cancer cell lines with low CAR expression. Intratumoral injection of Telomelysin-RGD into CAR-negative tumor xenografts in mice resulted in significant inhibition of tumor growth and long-term survival. These data suggest that fiber-modified Telomelysin-RGD exhibits a broad target ranges by increasing infection efficiency, although one needs to be

cautious about increased toxicity: since hematopoietic cell population such as dendritic cells can be efficiently infected with RGD-modified adenovirus [100].

A possible future direction for Telomelysin includes combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy, and new modalities such as antiangiogenic therapy. Since clinical activities observed by intratumoral injection of Telomelysin suggest that even partial elimination of the tumor could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human cancer. The combination of systemic chemotherapy and local injection of Telomelysin has been shown to be effective as described above [77]. In addition, we found that oncolysis induced by Telomelysin infection could be the most effective stimulus for immature dendritic cells to induce specific activity against human cancer cells. Therefore, Telomelysin can be effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that induces specific cytotoxic T-lymphocytes (CTL) for the remaining antigen-bearing tumor cells. Peri- or postoperative administration of Telomelysin may be also valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence or regrowth of tumors.

To our knowledge, no experimental viral agents that target human cancer including gene therapy products have been clinically approved in the world except in China. Advexin, which delivers normally functioning p53 tumor suppressor gene to cancer cells, will most likely be the first gene therapy drug approved in the US; the clinical development phase of Advexin, however, may be more than 10 years from the year the clinical study was initiated. The transition from phase I to phase III is also necessary for the development of Telomelysin. The recent surge in the approval rate for therapeutic monoclonal antibodies that were unsuccessful in the early 1980s is encouraging. Once one or more viral agents are approved in the US, the clinical development of oncolytic viruses is expected to move rapidly to the market.

The field of virotherapy is progressing considerably and is rapidly gaining medical and scientific acceptance. Although many technical and conceptual problems await to be solved, ongoing and future clinical studies will no doubt continue to provide important clues that may allow substantial progress in human cancer therapy.

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#### ABBREVIATIONS

AFP	=	$\alpha$ - Fetoprotein
CAR	=	Coxsackie's-adenovirus receptor
CCD	=	Cooled charged-coupled device
CMV	=	Cytomegalovirus
COX	=	Cyclooxygenase

CT	=	Computed tomography
CTL	=	Cytotoxic T-lymphocytes
EGF	=	Epidermal growth factor
ERE	=	Estrogen response element
FDA	=	Food and Drug Administration
FDG	=	<sup>18</sup> F-2-deoxy-D-glucose
GFP	=	Green fluorescent protein
HDAC	=	Histone deacetylase
HPV	=	Human papilloma virus
hTERT	=	Human telomerase reverse transcriptase
LC3	=	Light chain 3
MOI	=	Multiplicity of infection
MRI	=	Magnetic resonance imaging
MZF-2	=	Myeloid-specific zinc finger protein 2
NIH	=	National Institutes of Health
NSAID	=	Nonsteroidal anti-inflammatory drug
PET	=	Positron emission tomography
PSA	=	Prostate-specific antigen
RAC	=	Recombinant DNA Advisory Committee
TSA	=	Tricostatin A
WT1	=	Wilm's tumor suppressor 1

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消化器外科学

## GFP発現ウイルス製剤を用いた消化器癌微小転移の *in vivo* イメージングシステム

*In vivo imaging for micrometastasis of gastrointestinal cancer with tumor-specific GFP-expressing adenovirus*

近年増加を続ける癌患者の生存率や治療成績の向上には、早期発見、適格な悪性度の予知、適切な治療方針の決定などが重要な因子となる。より低侵襲な治療の導入は患者の生活の質(quality of life: QOL)を維持するためにも必要であり、手術の縮小化による低侵襲化をめざす際に有用な情報のひと

つに転移リンパ節の有無がある。生体内で微小癌組織や転移リンパ節を検出する試みは画像診断の分野で研究が進んでおり、たとえば positron emission tomography (PET)による生物学的診断や、ニューラルネットワークを駆使した画像解析などが検討されている。しかし、癌細胞へのターゲティ

ングの困難さから、術中 *in situ* での癌検出システムはいまだ開発されていない。

低侵襲手術のナビゲーションとして、センチネルリンパ節(sentinel node: SN)が注目されている。SNとは腫瘍から最初にリンパ流を受けるリンパ節であり、ここに最初の微小転移が生じるという仮説がSN理論である。乳癌では欧米を中心に大規模な臨床試験が開始されているが、その他の固形腫瘍にもこの考え方が通用するかについてはいまだ不明であり、その検証がはじまったところである。胃癌の単発リンパ節転移部位の解析から10%前後の skip 転移、すなわち第1群リンパ節を飛び越した第2群以遠リンパ節への初発転移が報告されており<sup>1)</sup>、これを根拠としてSNナビゲーションの危険性を唱える意見もある。

### テロメラーゼ活性を指標とする癌細胞の可視化

ウイルスは本来ヒトの細胞に感染して構造蛋白質を産生することで複製・増殖し、その細胞をさまざまな機序により破壊する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを標識する診断用製剤として用いることができる。染色体末端のテロメアを伸長する作用をもつ酵素テロメラーゼは85%以上のヒト悪性腫瘍でその活性の上昇が知られており<sup>2)</sup>、癌細胞ではその発現制御を行っているプロモーターのスイッチがオンになると考えられる(表1)。

Telomelysin® (OBP-301)は、幼児の“かぜ”症状の原因となる5型アデノウイルスの増殖に必須のE1遺伝子をテロメラーゼ構成成分であるヒトテロメラーゼ逆転写酵素(human telomerase reverse transcriptase: hTERT)遺伝子のプロモーターで駆動することで、癌細胞のみで選択的に増殖して細胞

表1 ヒト悪性腫瘍におけるテロメラーゼ活性

組織	テロメラーゼ陽性	組織	テロメラーゼ陽性
肺癌	84%	前立腺癌	83%
非小細胞肺癌	82%	膀胱癌	93%
小細胞肺癌	100%	腎癌	68%
頭頸部腫瘍	82%	Wilms 腫瘍	100%
食道癌	87%	網膜芽細胞腫	50%
胃癌	85%	脳腫瘍	49%
大腸癌	89%	神経芽細胞腫	94%
膵癌	95%	皮膚癌	83%
肝細胞癌	86%	基底細胞腫	95%
乳癌	86%	悪性黒色腫	86%
子宮癌		甲状腺癌	
子宮頸癌	93%	分化型	59%
子宮体癌	94%	未分化型	86%
卵巣癌	86%	肉腫	100%

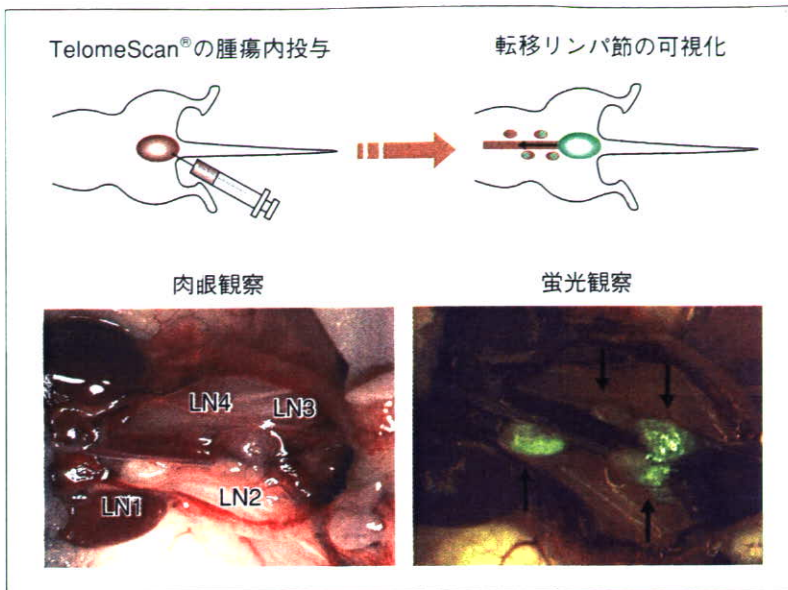


図 1 TelomeScan®によるリンパ節転移の*in vivo*イメージング<sup>7)</sup>

ヒト大腸癌細胞 HT29 をヌードマウスの直腸粘膜下に同所性に移植すると、直腸に腫瘍を形成するとともに 4~6 週間後に高率に傍大動脈リンパ節転移が認められる。TelomeScan®を直腸腫瘍に直接投与し、5 日後に開腹、蛍光励起して高感度 3CCD カメラにて観察したところ、4 個中 3 個のリンパ節で GFP 蛍光発現がみられた。この 3 個のリンパ節では、組織学的に微小転移が確認された。矢印：リンパ節。

死を誘導するように改変されたウイルス製剤である<sup>3,4)</sup>。抗癌剤としての Telomelysin® はアメリカ食品医薬品庁 (Food and Drug Administration : FDA) の承認のもと、2006 年 11 月よりアメリカで各種固形癌を対象とした第 I 相臨床試験が開始されており、その安全性と有効性のデータが集積されつつある。

TelomeScan® (OBP-401) は、Telomelysin® にオワンクラゲ由来の蛍光遺伝子 GFP (green fluorescence protein) を搭載したナノバイオ・ウイルス製剤であり、癌の診断および治療に有効であると考えられる<sup>5,6)</sup>。TelomeScan® の感染により、きわめて広範な癌細胞で GFP 蛍光の発現が観察され、一方、線維芽細胞をはじめとする正常細胞では GFP 陰性であった。また、ヌードマウスの背部皮下に移植したヒト悪性腫瘍内に TelomeScan® を投与したところ、24 時間後から 7 日以上長期にわたり癌組織に選択的な緑色蛍光発現が観察された<sup>7)</sup>。

### TelomeScan®による微小リンパ節転移の同定

ヌードマウスの直腸粘膜下にヒト大腸癌細胞 HT29 を移植すると、同所性に直腸腫瘍を形成するとともに、4~6 週間後に高率に傍大動脈リンパ節転移を生じる。このモデルにおいて、TelomeScan®を直腸腫瘍に直接腫瘍内投与し、5 日後に開腹、キセノン光で蛍光を励起して高感度 3 色冷却 CCD カメラにて観察した。GFP 蛍光を発したリンパ節を採取して最終的に病理組織学的に確認したところ、GFP 陽性リンパ節では高頻度に微小転移が検出された (図 1)。感度は、sensitivity 92.3%, specificity 86.6% であり、1 mm 以下の微小転移巣を蛍光 spot として同定することが可能であった<sup>7)</sup>。これらの結果は、原発腫瘍内に局所投与された TelomeScan® がリンパ流を經由して所属リンパ節へ拡散し、リンパ節内の微小転移巣で TelomeScan® が癌細胞に感染・増殖して選択的に GFP 蛍光を発し

たことを示唆している。また、TelomeScan® の複製・増殖はフロイドアジュバントの直腸粘膜投与で生じた炎症性のリンパ節腫大ではみられず、癌細胞に選択的に誘導されることが明らかとなった。

今後は TelomeScan® を標識薬剤とし、ペンプローブ型の高感度 GFP 蛍光検出装置を用いた微小癌組織診断用の外科手術ナビゲーション・システムを開発する。臨床的には内視鏡などのアクセスを用いて原発腫瘍内に局所投与することで、TelomeScan® はリンパ節内の微小転移巣で癌細胞に感染・増殖して選択的に GFP 蛍光を発するため、一定期間の後に開胸あるいは開腹で転移リンパ節を可視化することができる。この技術により、微小リンパ節転移を手術中にリアルタイムに検出してリンパ節郭清範囲を同定する低侵襲外科手術が可能となる。このシステムではセンチネルリンパ節生検と異なり、転移リンパ節そのものを同定できる点で確実性の面からきわめて実用的といえる。

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## 9. 癌のウイルス療法

*Oncolytic virotherapy*

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ウイルスによる腫瘍融解療法(Oncolytic virotherapy)は、新たな癌治療戦略として積極的に開発が進められている。遺伝子工学的技術の進歩と癌の分子病態解析の発展により、ウイルスの細胞傷害活性を癌細胞に標的化することが可能となってきた。理論的根拠に基づいた癌選択性の確保と正常細胞での毒性軽減は、ウイルス製剤の臨床応用を現実のものとしてきている。本稿では、著者らが開発をすすめるテロメラーゼ依存性腫瘍融解ウイルス Telomelysin を中心に、癌のウイルス療法の可能性を概説する。

key  
word

アデノウイルス/テロメラーゼ/臨床試験

### はじめに

ウイルスは、本来ヒトの細胞に感染して増殖複製し、その細胞をさまざまな機序により破壊する。1900年代の初めより、癌細胞でその殺細胞効果を期待して野生型のウイルスを用いた癌治療が試みられてきた<sup>1)</sup>。子宮癌や黒色腫に対する狂犬病ウイルスの投与や、コクサッキーB型ウイルス、ミクソウイルス、アルポウイルスなどによる固形癌の治療が行われてきた。1974年には、進行癌患者へのムンプスウイルス投与の本邦での研究成果が報告されている<sup>2)</sup>。しかし、これらのウイルスはあらゆる細胞で増殖能を有する野生型であったため、毒性などにより一般的な治療としては使用されるには至らなかった。

最近の遺伝子工学の進歩により、ウイルスゲノムを改変し、その安全性を高めたり特殊な機能を増強することが可能となってきた。最初の試みは、ウイルスゲノムの一部を欠損させることで増殖性を押え、治療遺伝子を発現させることで安全性と機能を確保した。このウイルスベクターを用いた「遺伝子治療」がヒトに応用されてから、すでに10年以上が経過し

ている。多くの非増殖型ウイルスベクターが臨床応用され、特定の患者群に対しては有用性が認められた<sup>3)</sup>。しかし、*in vivo*における標的組織への遺伝子導入効率の限界などから、必ずしも前臨床試験で期待された臨床効果が認められているとはいいがたい。

そこで、ウイルスの増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを傷害する治療用製剤として用いようとする試みがなされるようになってきた<sup>4)</sup>(図1, 表1)単純ヘルペスウイルスやワクシニアウイルスなどの改変が行われており、臨床応用も積極的に進んでいる。しかし、アデノウイルスがその構造が最もよく研究されているウイルスの一つであり、非増殖型のものが多くの遺伝子治療プロトコルで使用されることによって、その安全性に関する情報が蓄積されてきている。

本稿では、テロメラーゼ活性依存性に癌細胞で選択的に増殖して細胞死を誘導するアデノウイルスの開発の現況について紹介し、その抗腫瘍医薬品としての臨床応用の可能性を考察する。



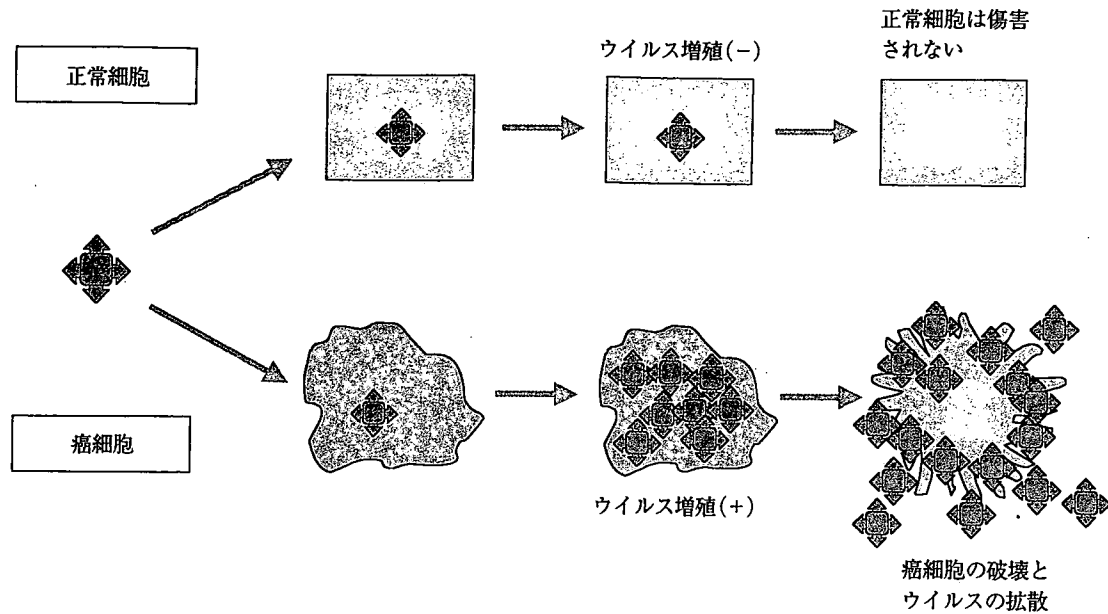


図1 癌細胞での選択的なウイルス増殖と細胞死誘導(文献3より)

表1 癌のウイルス療法の臨床応用

ウイルス & 薬剤名	作用機序	対象疾患	臨床試験
<b>アデノウイルス</b>			
Onyx-015	E1B 55kd 欠損	頭頸部癌 卵巣癌 原発性 & 転移性肝臓癌 膵臓癌 大腸癌	II-III I I-II I I-II
Ad5-CD/TKrep	E1B 55kd 欠損 + HSV-tk/CD 遺伝子挿入	前立腺癌	I
CV706	PSA プロモーターによる E1A 遺伝子制御	前立腺癌	I-II
CV787	Probasin プロモーターで E1A, PSA プロモーターで E1B 制御	前立腺癌	I-II
OBP-301 (Telomelysin)	hTERT プロモーターで E1A および E1B 遺伝子制御	各種固形癌	I
<b>ヘルペスウイルス</b>			
G207	$\gamma$ 34.5 欠損と ICP6 の機能欠損	悪性グリオーマ	I-II
NV1020	$\gamma$ 34.5 欠損と内因性 tk 欠損と外来性 tk 遺伝子挿入	転移性肝臓癌	I
OncoVEX	$\gamma$ 34.5 欠損と ICP47 領域への GM-CSF 遺伝子挿入	乳癌 頭頸部癌 悪性黒色腫	I-II I-II I-II
<b>ワクシニアウイルス</b>			
Vaccinia-oncolysate	腫瘍融解産物とウイルスによる免疫賦活	悪性黒色腫	III
Vaccinia-GM-CSF	GM-CSF 遺伝子挿入	悪性黒色腫	I
Vaccinia-CEA	CEA 遺伝子発現による抗原提示	CEA 産生腫瘍	I
Vaccinia-PSA	PSA 遺伝子発現による抗原提示	前立腺癌	I
<b>ニューキャッスル病ウイルス</b>			
PV701	弱毒化株	各種固形癌	I
MTH-68/H	弱毒化株	グリオブラストーマ	I
<b>レオウイルス</b>			
Reolysin	自然株	頭頸部癌	I
<b>ムンプスウイルス</b>			
MV-CEA	MV-Edm 株に可溶性 CEA 挿入	卵巣癌	I

## アデノウイルスの特徴と制限増殖能の分子機構

ヒトのアデノウイルスはエンベロープを持たない 30-38kD サイズの二重鎖 DNA ウイルスであり、41 種の亜型が存在し、6 群に分類されている。遺伝子導入用ベクターの基本骨格としてよく用いられるアデノウイルス 5 型は、幼児期に気道感染によりいわゆる「かぜ」症状を起こす原因ウイルスの一つであり、米国では30年以上の間、約100万人の兵士に対しワクチンとしてアデノウイルスが投与され、その後重篤な副作用の報告もなかったという実績を持つ。

アデノウイルスゲノムはその構造が詳細に解析されており、ウイルスの複製増殖のきわめて初期(immediate-early: IE)に働く遺伝子群、初期(early: E)に働く遺伝子群、および後期(late: L)に関与する遺伝子群に分けられる。現在、アデノウイルスに癌細胞に特異的な増殖機能を発揮させるために、大きく二つの方法が開発されている。

最初の試みは、アデノウイルスの初期遺伝子に特定の変異あるいは欠失を加えることにより癌細胞の生物学的な特殊性に基づいた制限増殖性を期待する方法であり、E1B 初期遺伝子の 55kD を欠損した 2 型および 5 型のキメラタイプの変異アデノウイルスである Onyx-015 (dl1520) が代表的である<sup>4)</sup>。本来、E1B-55kD 蛋白質は癌抑制遺伝子産物である p53 蛋白質に結合し、その機能を不活化する働きを担って

いる。したがって、Onyx-015 は、正常な p53 機能を持つ細胞では p53 によるウイルスの複製増殖の抑制を制御することができない。

一方、p53 機能を喪失している癌細胞では、E1B-55kD が作用する必要がなく、Onyx-015 は複製増殖により細胞融解を誘導することが可能となる。しかし、その後の研究により、Onyx-015 の増殖能は必ずしも p53 機能の有無に因らないことが明らかになっており<sup>5)</sup>、またヒトの正常細胞での増殖複製の可能性も示唆されている<sup>6)</sup>。Onyx-015 と類似の腫瘍融解ウイルス (Oncolytic virus) である H101 は、中国食品医薬品局 (State Food and Drug Administration: sFDA) の承認を受け、すでに市場に出ている<sup>7)</sup>。

癌選択性を持たず第二の試みは、腫瘍特異的および臓器特異的なプロモーターによる初期遺伝子の転写制御をメカニズムとして、癌細胞特異的あるいは特定の臓器由来の癌細胞に特異的な制限増殖性を付加する方法である。この際、さまざまな発生母地を持つ広い範囲の癌に適応するためには、より汎用性を有するプロモーターを用いる必要がある。

## テロメラーゼ活性と *hTERT* 遺伝子

染色体 DNA 末端の短い塩基配列 (TTAGGG) の繰り返しで構成されるテロメアは、細胞増殖に伴いしだいに短縮し細胞に老化 (replicative senescence) を引き起す。このテロメアの短縮は発癌の抑制機構であり、前癌状態にある細胞が老化に陥り死滅するこ

表 2 ヒト悪性腫瘍におけるテロメラーゼ活性

組 織	テロメラーゼ陽性	組 織	テロメラーゼ陽性
肺癌	84%	前立腺癌	83%
非小細胞肺癌	82%	膀胱癌	93%
小細胞肺癌	100%	腎臓癌	68%
頭頸部腫瘍	82%	ウィルムス腫瘍	100%
食道癌	87%	網膜芽細胞腫	50%
胃癌	85%	脳腫瘍	49%
大腸癌	89%	神経芽細胞腫	94%
膵臓癌	95%	皮膚癌	83%
肝細胞癌	86%	基底細胞腫	95%
乳癌	86%	悪性黒色腫	86%
子宮癌		甲状腺癌	
子宮頸癌	93%	分化型	59%
子宮体癌	94%	未分化型	86%
卵巣癌	86%	肉腫	100%

とで癌化が阻止されている。逆に、無制限の増殖能を有する癌細胞はテロメアを維持する分子機構を獲得しており、代表的なものがテロメラーゼの活性化である。テロメラーゼは、染色体の3末端にTTAGGG配列を伸長しテロメア長を保つ作用を持つリボ核酸蛋白酵素であり、触媒サブユニットhTERT (human telomerase reverse transcriptase)と鋳型となるRNAサブユニット(hTR)から構成される。テロメラーゼ活性はhTERT遺伝子発現レベルと相関し、またhTERT遺伝子導入によりテロメラーゼ活性を誘導することができることから、hTERT分子がテロメラーゼ活性を制御していると考えられる<sup>8)</sup>。テロメラーゼは、きわめて多くの癌細胞でその活性の上昇が明らかになっており<sup>9)</sup>(表2)、癌細胞ではhTERT遺伝子の発現制御を行っているhTERTプロモーターのスイッチがオンになると考えられる。

### テロメラーゼ特異的腫瘍融解アデノウイルスの構造と機能

前立腺癌に特異的なPSA<sup>10)</sup>をはじめとして、AFP<sup>11)</sup>やMUC-1<sup>12)</sup>などさまざまなプロモーターによる癌特異的に増殖するアデノウイルスが開発されており、それぞれのプロモーター機能に対応する癌細胞においてはその有効性が示されている。しかし、より広範な癌を対象とするために、著者らはアデノウイルスの増殖に必要なE1A遺伝子とE1B遺伝子をIRES配列で結合した発現カセットをhTERTプロモーターにより選択的に発現するテロメラーゼ特異的腫瘍融解ウイルスTelomelysin(開発コード:OBP-301)を作成した<sup>13)</sup>。

多くの制限増殖型アデノウイルスがE1A遺伝子のみを選択的プロモーターで制御しているのに比べて、TelomelysinではE1AおよびE1BをいずれもhTERTプロモーターの制御下に置くことで、より癌細胞での特異性が確保できている。実際に、Telomelysin感染後3日までに、各種癌細胞においては $10^5 \sim 10^8$ 倍のウイルス複製増殖が認められたが、正常細胞では100~1,000倍に抑えられていた。肺癌、大腸癌、胃癌、食道癌、頭頸部癌、乳癌、肝癌、膵癌、前立腺癌、子宮頸癌、卵巣癌などのヒト由来各種癌細胞では、1-10 multiplicity of infection (MOI)

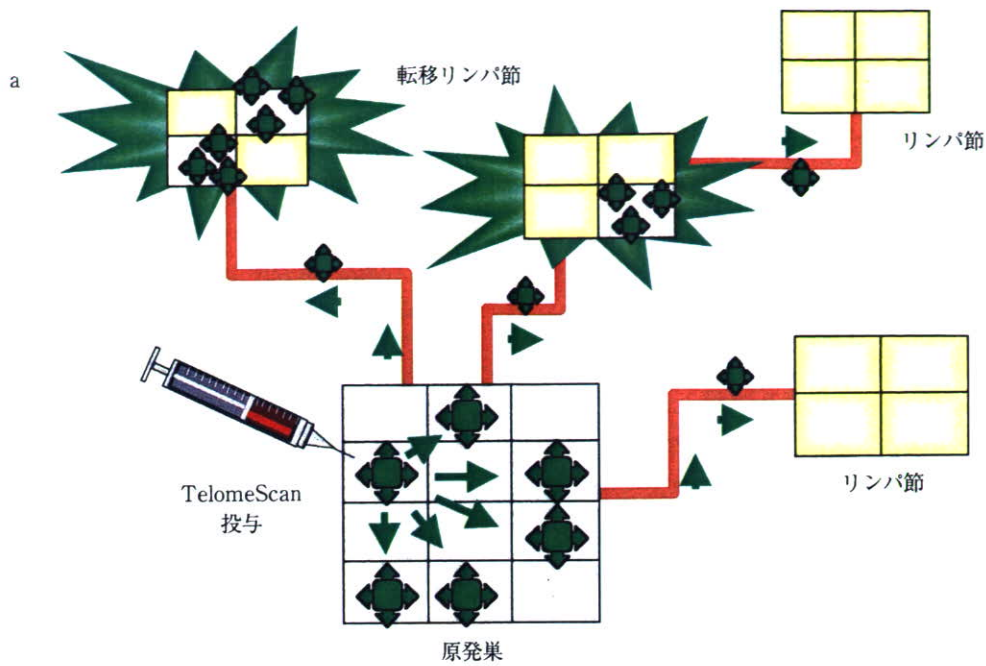
のTelomelysin感染で3~5日以内にcytopathic effect (CPE)が誘導され完全な細胞死が観察された。ヌードマウス背部皮下に移植したヒト肺癌腫瘍に、低濃度のTelomelysinを腫瘍内局所投与したところ、無治療の腫瘍や非増殖型のコントロール・アデノウイルスの投与に比較して有意な増殖抑制が認められ、さらにTelomelysinは血中を循環し、遠隔部位の腫瘍内でも増殖していることがDNA-PCR解析やE1A蛋白質に対する免疫染色などにより確認された。これらの結果は、原発腫瘍内投与したTelomelysinによる微小転移巣の治療の可能性を示唆している。

### Armed(武装化)Telomelysinの開発

Telomelysinのウイルスゲノムにさまざまな機能遺伝子を組み込むことで、特殊機能の付加や抗腫瘍活性の増強を期待することができる。

TelomeScan(OBP-401)は、Telomelysinを基本骨格としてオワンクラゲ由来の蛍光遺伝子GFP(Green Fluorescence Protein)遺伝子をウイルスゲノムに組み込んでおり<sup>14)15)</sup>、生体内で癌組織を可視化する診断用医薬品、あるいはナビゲーション・ツールとしても使用可能である。TelomeScanは癌細胞で選択的に増殖してGFP緑色蛍光を発するため、*in vitro*では蛍光顕微鏡下に、またマクロでは高感度3CCDカメラを用いた蛍光観察システム下に癌組織を検出することが可能である。実際に、ヒト癌細胞をヌードマウスの胸腔内に移植した胸膜播種モデルにおいて、TelomeScanの胸腔内投与により肉眼的には確認できなかった微小胸膜播種巣の選択的な可視化が可能であった<sup>16)</sup>。

また、生体内で癌組織や転移リンパ節を検出する試みは画像診断の分野で研究が進んでいるが、手術中に直接検出・診断するシステムはいまだ開発されていない。手術の縮小化による低侵襲化を目指す場合にほしい情報の一つに転移リンパ節の有無があり、それを知る方法としてTelomeScanが活用できる。ヒト大腸癌細胞とヌードマウスを用いた同所性直腸癌モデルにおいて、TelomeScanを直腸腫瘍に直接投与し、5日後に開腹、キセノン光で蛍光を励起して高感度3CCDカメラにて観察したところ、大動脈周囲のGFP陽性リンパ節では高頻度に微小転移が検



肉眼観察

蛍光観察

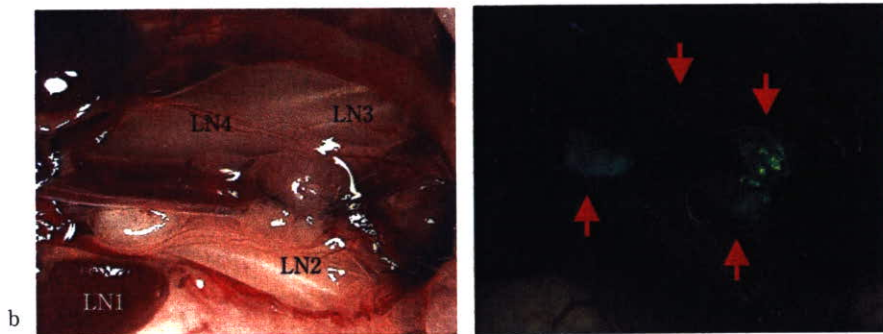


図2 TelomeScanによるリンパ節転移の *in vivo* イメージング

- a: 原発巣に局所投与された TelomeScan は、リンパ流に乗って所属リンパ節に到達する。そこで、微小リンパ節転移が存在すれば、ウイルスの複製・増殖とともに GFP 蛍光を発するため、転移リンパ節は緑色蛍光で可視化される。
- b: ヒト大腸癌細胞 HT29 をヌードマウスの直腸粘膜下に同所性に移植すると、直腸に腫瘍を形成するとともに 5~6 週間後に高率に傍大動脈リンパ節転移が認められる。TelomeScan を直腸腫瘍に直接投与し、5 日後に開腹、蛍光励起して高感度 3CCD カメラにて観察したところ、4 個中 3 個のリンパ節で GFP 蛍光発現がみられた。この 3 個のリンパ節では、組織学的に微小転移が確認された。矢印: リンパ節。

出された<sup>17)</sup>(図2)。実際の臨床では、TelomeScanを手術前に内視鏡などで癌局所に注入し、高感度蛍光感知プローブにより転移陽性リンパ節を直接術中にリアルタイムに同定し、切除範囲のナビゲーションとすることが可能である。

OBP-405 は、Telomelysin の標的細胞への感染性を増強するために、細胞表面のインテグリンに親和性を有する RGD(Arg-Gly-Asp)モチーフをファイバーに組み込んだ改変 Telomelysin である<sup>18)</sup>。OBP-

405 はコクサッキー・アデノウイルス受容体(Coxsackie-adenovirus receptor: CAR)非依存性にインテグリンを介して標的細胞に感染することができ、CAR 陰性のヒト悪性腫瘍細胞でも顕著な抗腫瘍効果を発揮することが可能であった。CAR 発現低下により Telomelysin に耐性となった癌に対しても OBP-405 は抗腫瘍効果を発揮すると推測され、Telomelysin の back up として有用であると思われる。