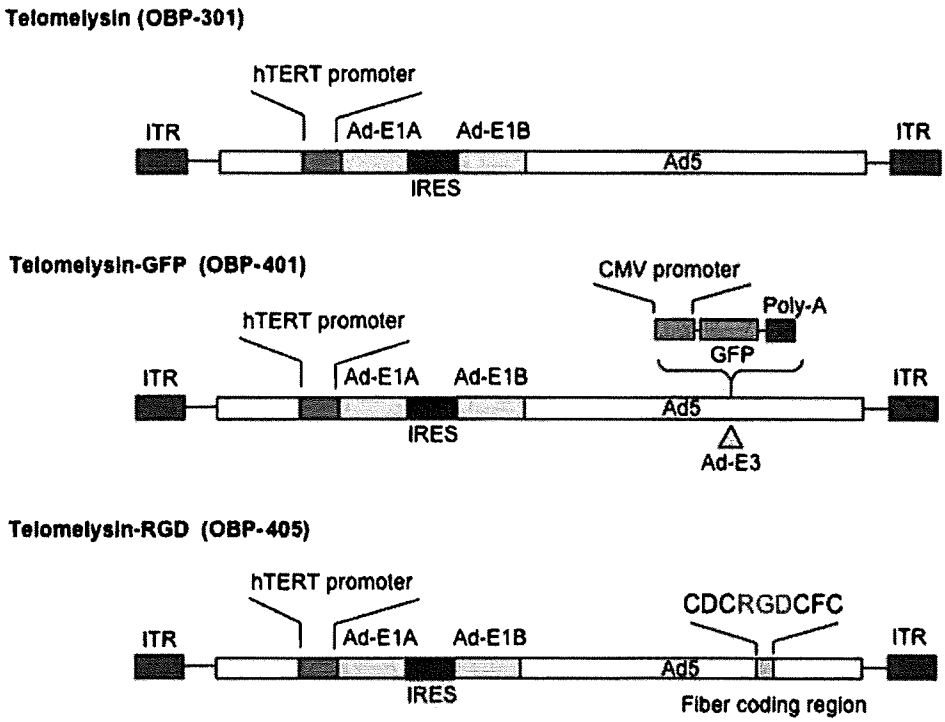

Fig. 2. Schematic DNA structures of telomerase-specific oncolytic viruses. Telomelysin (OBP-301) has *E1A* and *E1B* genes linked with an *IRES*, driven by the human telomerase reverse transcriptase (*hTERT*) promoter. A variant of OBP-301 was constructed that has the green fluorescent protein (*GFP*) 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.

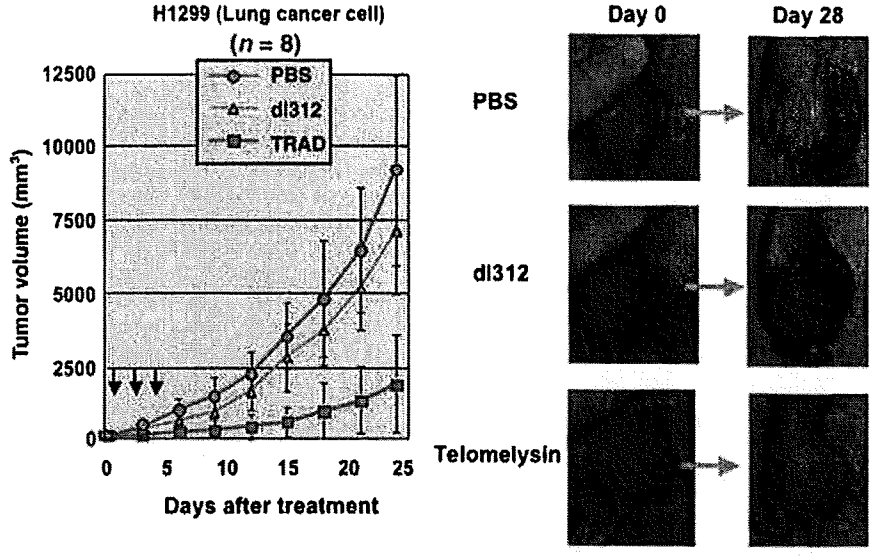

Fig. 3. *In vivo* effect of Telomelysin on tumorigenesis. Lung cancer H1299 cells were inoculated to the flank of nu/nμ mice. Mice bearing palpable tumors with a diameter of 5–6 mm received intratumoral injection of 10^7 PFU of Telomelysin or replication-deficient adenovirus (dL312) or phosphate-buffered 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.[105]

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

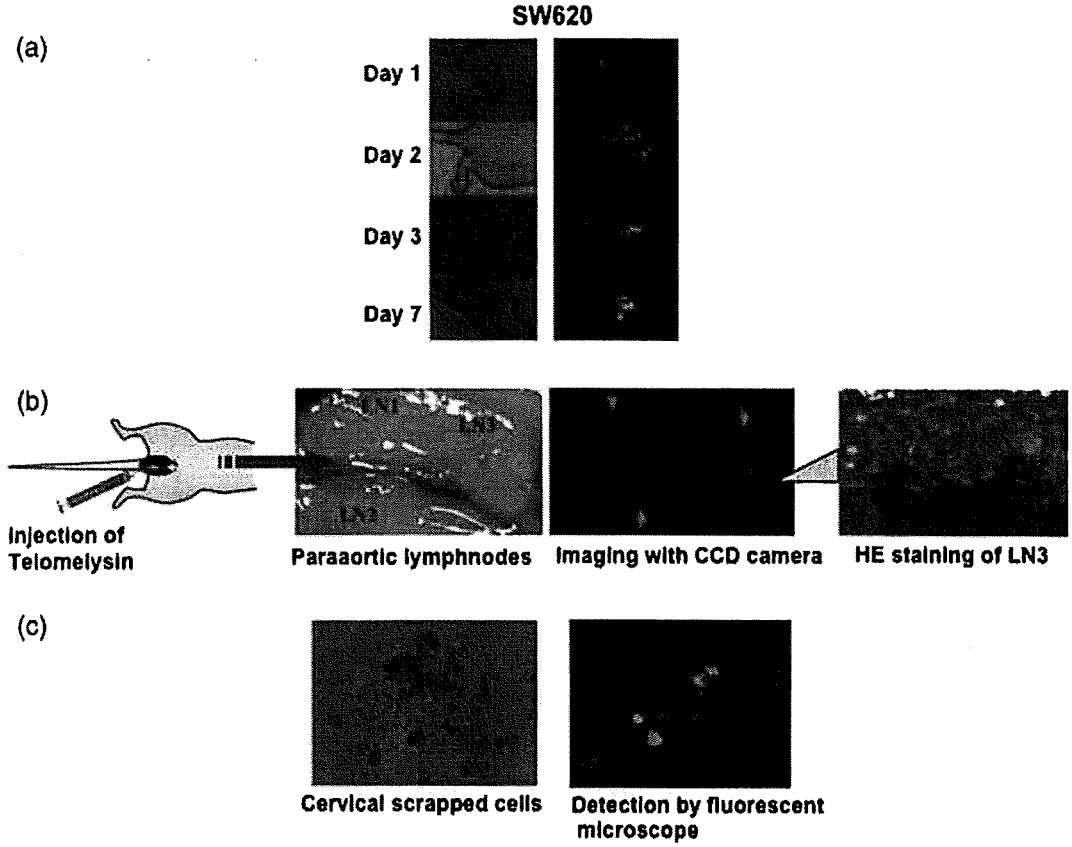

Fig. 4. Diagnostic utility of TelomeScan. (a) Visualization of tumor by the injection of TelomeScan. Subcutaneous tumor xenografts by colon cancer (SW620) were directly injected by TelomeScan at concentrations of 8×10^6 PFU. The green fluroescent protein (GFP) fluorescence intensity was monitored for 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

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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1568-0096/07 $50.00+.00

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, tricostatin A (TSA), could induce significant activation of hTERT mRNA

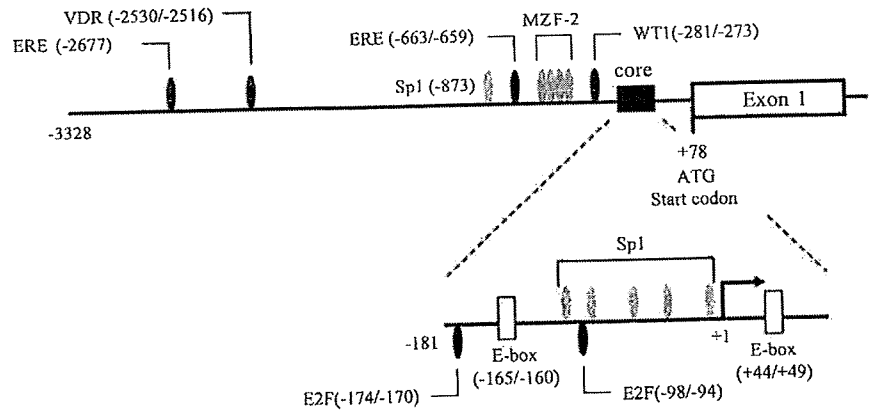

Fig. (1). Scheme of the proximal promoter of hTERT. Putative protein binding sites for various transcription factors are indicated.

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 α- 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

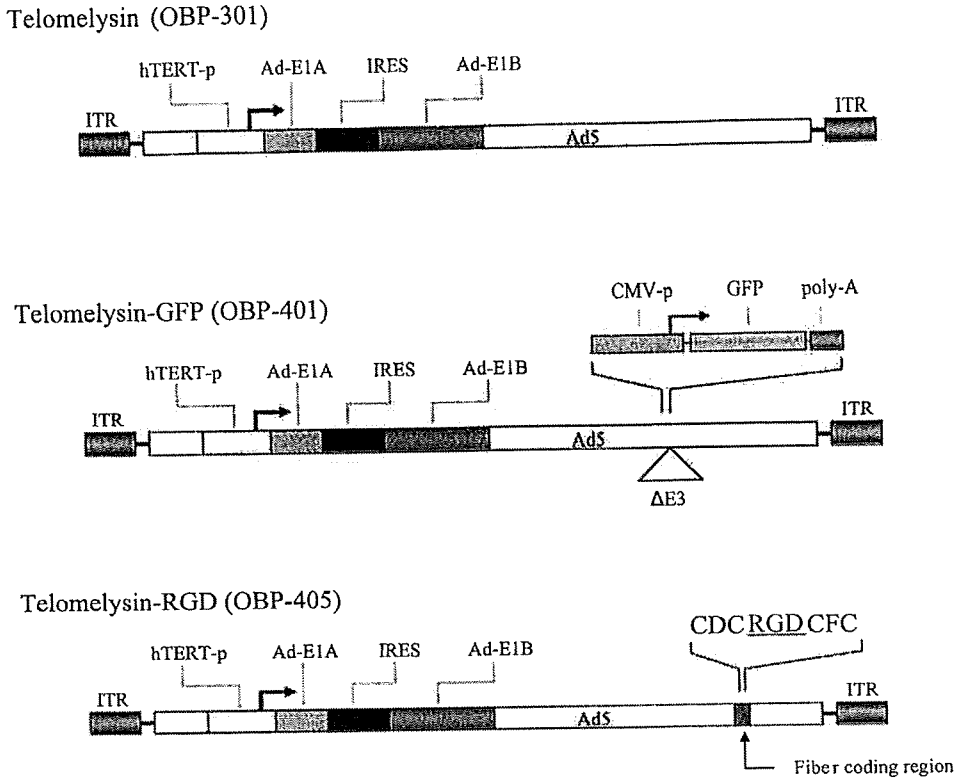

Fig. (2). Schematic DNA structures of telomerase-specific oncolytic viruses. Telomelysin (OBP-301), in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an IRES. Telomelysin-GFP (OBP-401) is a telomerase-specific replication-competent adenovirus variant, in which 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]. Macro-scopically, 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 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

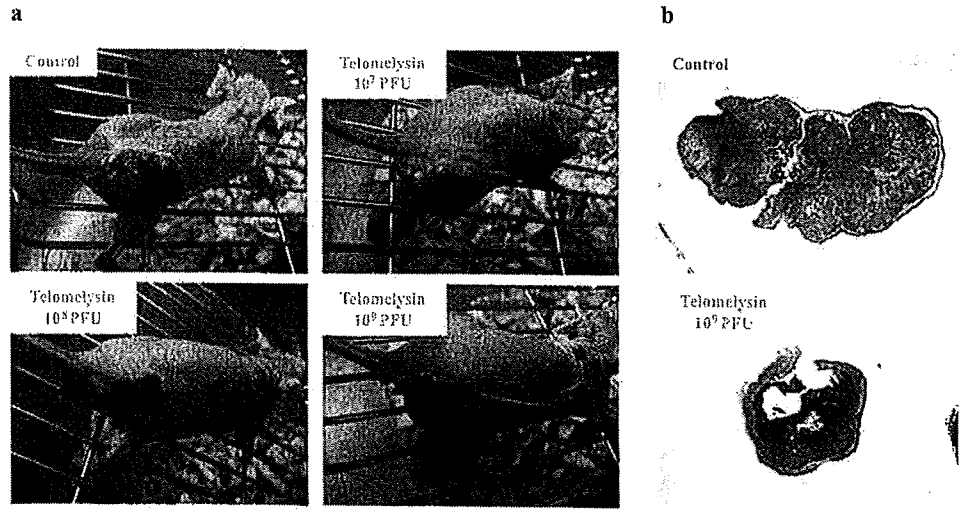

Fig. (3). Antitumor effect of intratumorally injected Telomelysin against established flank SW620 xenograft tumors in *nu/nu* mice. (a) Macroscopic appearance of tumors 15 days after treatment with various concentrations of Telomelysin. (b) Tumors were dissected 15 days after viral injection and paraffin 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 Coxsackie'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}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

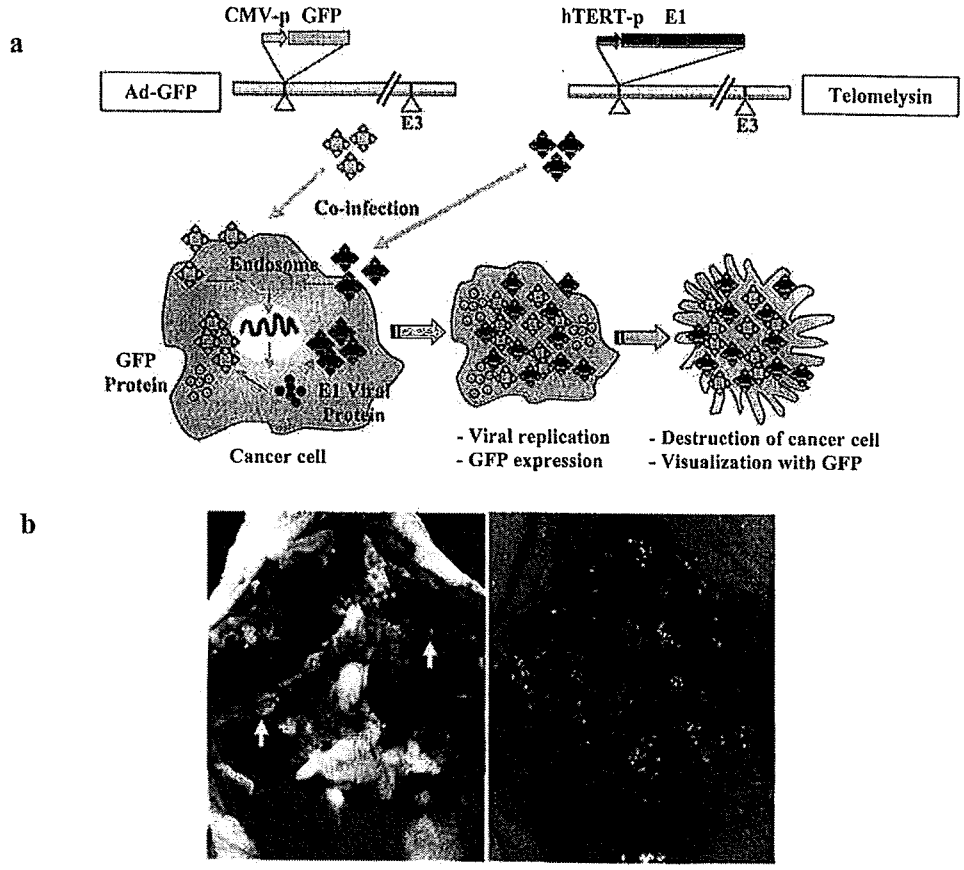

Fig. (4). (a) Concept of selective visualization of tumor cells with Ad-GFP and Telomelysin. (b) Internal images of pleural dissemination visualized by intrathoracic injection of Ad-GFP and Telomelysin. Female BALB/c *nu/nu* mice received intrathoracic implant with A549 human lung tumor cells. Five days 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%,

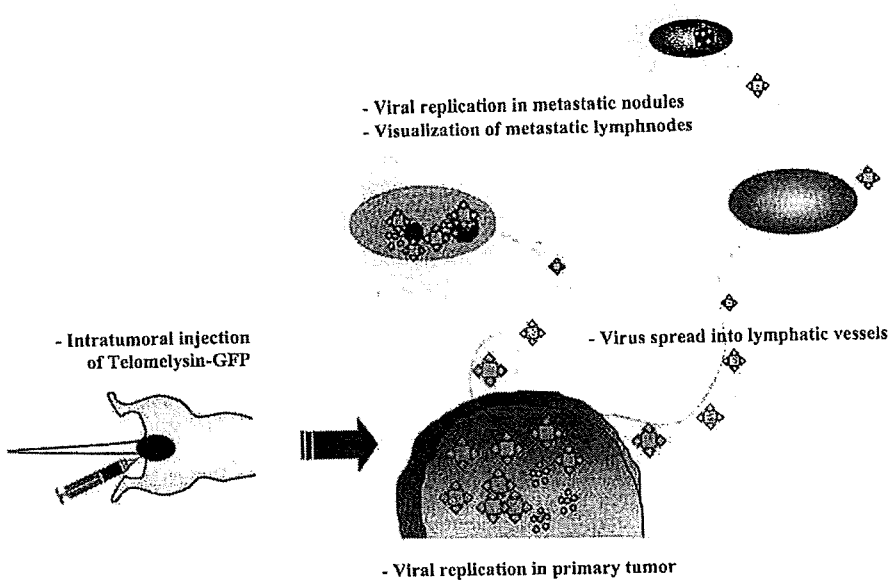

Fig. (5). Concept of selective visualization of lymph node metastasis with Telomelysin-GFP.

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.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture, Japan; and Grants from the Ministry of Health and Welfare, Japan. We thank Drs. Hitoshi Kuwamura and Katsuyuki Nagai (Oncolys BioPharma, Inc.) for helpful discussions at all stages of this work.

ABBREVIATIONS

AFP = α- 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 = ^{18}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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Received: June 14, 2006 Revised: July 05, 2006 Accepted: July 12, 2006

Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma

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(Received November 21, 2008/Revised April 10, 2009/Accepted April 19, 2009/Online publication June 2, 2009)

The relationship between overexpression of glypican (GPC)-3 that is specific for hepatocellular carcinoma (HCC) and the prognosis has not yet been clarified. We attempted to determine the expression profile of GPC3 in association with the clinicopathological factors by immunohistochemical analysis in HCC patients and investigated the potential prognostic value of GPC3 by comparing the survival rate between the GPC3-positive and GPC3-negative HCC patients. Primary HCC tissue samples (n = 107) obtained from patients who had undergone hepatectomy between 2000 and 2001 were analyzed. GPC3 expression was less frequently observed in well-differentiated HCC than in moderately and poorly differentiated HCC, the difference in the frequency being statistically significant. GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, P = 0.031). Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period. No deaths were noted in the GPC3-negative HCC patients among the 71 (88.7%) patients with moderately and poorly differentiated HCC. Multivariate analysis identified GPC3 expression (P = 0.034) as an independent prognostic factor for the overall survival. We showed that GPC3 expression is correlated with a poor prognosis in HCC patients. (*Cancer Sci* 2009; 100: 1403–1407)

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked as the third most common cause of cancer-related death worldwide. HCC is generally associated with a poor prognosis, the 5-year survival rate after surgery has been reported to be as low as 25–39%, and systemic therapy with cytotoxic agents provides only marginal benefit.[1] Even in those patients in whom the tumor has been successfully removed, the 2-year recurrence rate can be as high as 50%.[2,3] Several clinicopathological factors including poor levels of differentiation of the cancer cells, large size of the tumor, portal venous invasion, and intrahepatic metastasis have been shown to contribute to the poor prognosis in patients of HCC. Despite the critical need for better methods for the diagnosis and treatment of HCC, the mechanisms underlying the development of HCC remain unclear.

Glypican (GPC)-3 was discovered as a potential serological and histochemical marker that is specific for HCC. GPC3 is a member of the glypican family and belongs to a group of heparan sulfate proteoglycans bound to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor.[4] In mammals, this family comprises six members, GPC1 to GPC6. GPC are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors, and bone morphogenetic proteins.[5–9] Depending on the context, their functions exerted may either be stimulatory or inhibitory through these pathways. GPC3 has been detected in the placenta and fetal liver, but not in other adult organs. During hepatic carcinogenesis, GPC3 appears in the HCC tissue and is released into the serum.[10–12] In addition, its expression has also been reported in melanoma.[13–15]

A dramatic elevation of GPC3 expression has been reported in a large proportion of HCC, as determined by cDNA microarray analysis, whereas its expression has been shown to be less frequent in preneoplastic or entirely absent in non-neoplastic liver tissue.[16–18] This has led to the notion that GPC3 may have diagnostic usefulness as a marker of differentiation or a specific tumor marker in the case of HCC. However, until now, the relationship between GPC3 overexpression and the prognosis of HCC has not been clarified.

In the present study, we attempted to determine the tumor expression profile of GPC3 in association with clinicopathological factors in HCC patients by immunohistochemical analysis. We also investigated the potential prognostic value of GPC3 by analyzing the survival rate of GPC3-positive and GPC3-negative HCC patients. By elucidating the association between the GPC3 expression level in HCC tumors and the survival rate of the patients, we concluded that the GPC3 expression level is correlated with a poor prognosis in HCC patients.

Materials and Methods

Patients and tumor tissue samples. Primary HCC tissue samples (n = 107) were obtained from patients who underwent hepatectomy at the National Cancer Center Hospital East between 2000 and 2001. The histological types were assigned according to the criteria of the World Health Organization classification. Liver tissue sections prepared from the surgically resected tumors and adjacent parenchyma fixed in 10% formalin and embedded in paraffin were retrieved from the files of the Department of Pathology at our institution.

Immunohistochemical staining. Sections 6 μm thick were prepared from the paraffin-embedded blocks. The sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, sections were heated in 10 mM citrate buffer (pH 6.0) with microwave at 95°C for 15 min. The slides were then allowed to cool down, and the prediluted primary monoclonal anti-GPC3 antibody (dilution 1 : 300; Biomosaics, Burlington, VT, USA) was added to cover each slide, and the slides were incubated for 2 h at room temperature. Thereafter, the slides were washed three times in TBS–Tween 20 for 5 min each. Mouse Envision Polymer-horseradish

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doi: 10.1111/j.1349-7006.2009.01206.x

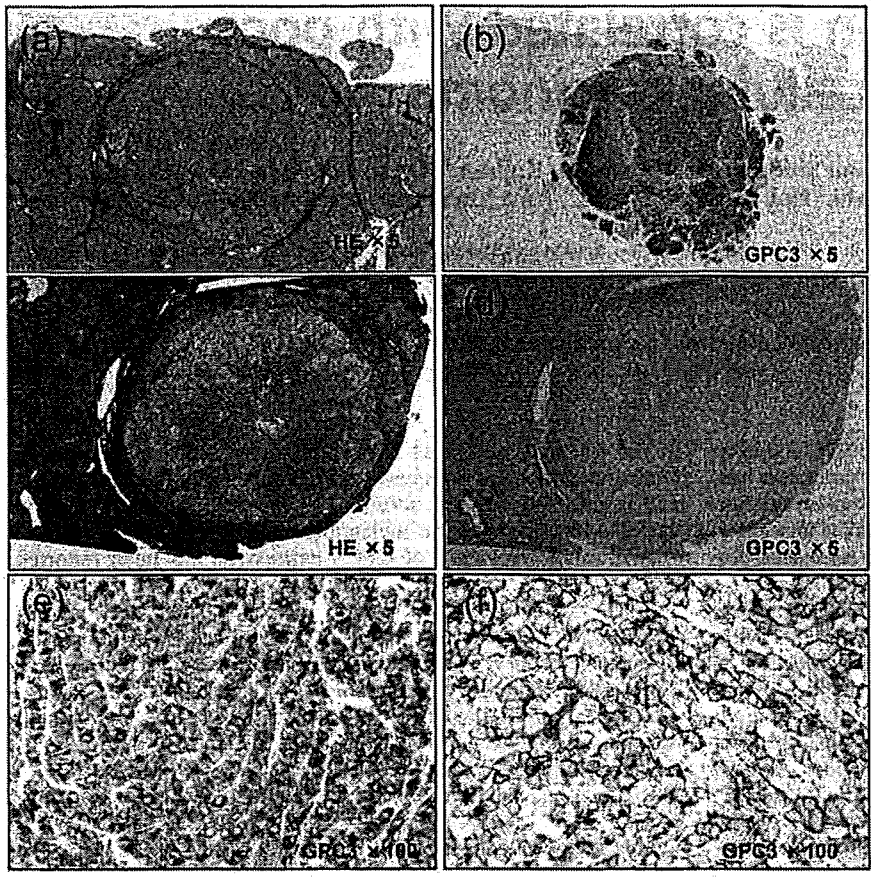

Fig. 1. Glypican (GPC)-3 expression and localization is hepatocellular carcinoma (HCC)-specific. (a,c) Microscopic view of a HE-stained sections of resected HCC. (b,d) HCC sections were stained for GPC3 expression with anti-GPC3 monoclonal antibody. (e) HCC displays prominent bile-canalicular immunostaining. (f) Membranous and cytoplasmic staining of liver tumor cells are shown.

peroxidase (DakoCytomation, Carpenteria, CA, USA), was used as the secondary antibody for 30 min at room temperature followed by three washes in TBS–Tween 20 for 5 min each. Finally, the visualization signal was developed by the addition of 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation) to each slide, followed by incubation for 2 min. Slides were then washed in distilled water, counterstained with hematoxylin, and dehydrated.

For the immunohistochemical analysis of GPC3, we evaluated only the area of GPC3-positive staining in one slide in each patient, including the HCC lesion and adjacent non-cancerous lesion. At first, to analyze GPC3 expression, the results of immunohistochemical staining were classified according to the area of GPC3-positive staining cells as follows: −, negative (<10%); +/−, weakly positive (10–30%); and +, positive (>30%). Finally, in this study, we classified two groups between GPC3-negative (<10%) and GPC3-positive (>10%). The expression of GPC3 was judged to be positive when the percentage of immunoreactive cells was semiquantitatively assessed as being ≥10% in focal lesions. The slides were examined independently by two observers (H. Shirakawa and T. Nakatsura) and then collectively by a pathologist (M. Kojima).

Analysis of the correlation of GPC3 expression with various clinicopathological factors. The correlation of GPC3 expression with various clinicopathological factors was analyzed. Overall survival was calculated from the date of surgery to the date of death.

Statistical analysis. The differences in the level of GPC3 expression were tested by the χ^2-test and the means of each subgroup were compared using Student's *t*-test. Survival analyses were carried out according to the Kaplan–Meier method and the differences were assessed using the log-rank test. Follow-up time was censored if the patient was lost to follow up. Cox proportional-hazards analysis was used for univariate and multivariate analyses to explore the effects of the variables on survival. *P*-values of less than 0.05 were considered to be significant.

Results

Glypican-3 expression in HCC. In order to characterize the expression of GPC3 in HCC, 107 surgical specimens were analyzed immunohistochemically. The mean and median follow-up period were 3.4 ± 2.0 years and 3.5 years respectively. GPC3 expression was detected in 87 of the surgically resected tumor specimens (81.3%) (Fig. 1a,b), but not in the remaining 20 specimens (18.7%) (Fig. 1c,d). In most of the GPC3-positive cases, the protein expression was localized mainly in the cellular cytoplasm (Fig. 1e) with some amount detected on the cell membrane (Fig. 1f). The results of the immunohistochemical analysis were evaluated in relation to the pathological findings and follow-up data. There was no correlation between GPC3 expression and any of the clinicopathological features, except that the GPC3 expression increased with increasing degree of dedifferentiation of the cancer cells (Table 1). GPC3 expression was less frequently observed in well-differentiated HCC than in moderately or poorly differentiated HCC; the difference in frequency was statistically significant. Thus, an increase in GPC3 expression was correlated with increasing aggressiveness of the cancer cells, which was accompanied by dedifferentiation of the cells.

Correlation between GPC3 expression and patient survival. In order to determine the prognostic value of GPC3, the overall survival was compared between GPC3-positive and GPC3-negative HCC patients. The GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, $P = 0.031$; Fig. 2a). After surgery,

doi: 10.1111/j.1349-7006.2009.01206.x

Table 1. Correlation between glypican (GPC)-3 expression and clinicopathological features of patients with hepatocellular carcinoma

Variable	GPC3 expression		P-value
	Positive (n = 87)	Negative (n = 20)	
Age (years) (mean ± SD)	63.6 ± 9.7	60.2 ± 11.8	0.169
Sex (male/female)	67/20	18/2	0.321
HBsAg status (positive/negative)	26/61	3/17	0.283
HCV status (positive/negative)	50/37	12/8	0.999
ICG R15 (%) (mean ± SD)	15.9 ± 8.1	15.5 ± 7.6	0.823
AFP (ng/mL) (mean)	6710	463	0.198
PIVKA-II (mAU/mL) (mean)	7370	5900	0.823
Tumor occurring (primary/recurrence)	64/23	16/4	0.753
Number of tumor (solitary/multiple)	64/23	11/9	0.172
Resection procedure (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	22/65	7/13	0.378
Operation time (min.) (mean ± SD)	310 ± 165	263 ± 119	0.248
Intraperative blood loss (mL) (mean)	2910	1500	0.356
Perioperative transfusion (present/absent)	45/42	9/11	0.767
Tumor size (mm) (mean ± SD)	54.7 ± 41.9	53.0 ± 31.2	0.861
Histological tumor differentiation (well/moderately and poorly)	6/81	6/14	0.032
pStage (UICC) (I/II/III)	35/41/11	6/10/4	0.577
Portal vein involvement (present/absent)	39/48	8/12	0.885
Hepatic vein involvement (present/absent)	9/78	1/19	0.750
Bile duct involvement (present/absent)	11/76	1/19	0.557
Intrahepatic metastasis (present/absent)	18/69	6/14	0.545
Non cancerous tissue (cirrhosis/non-cirrhosis)	36/51	4/16	0.075
Postoperative recurrence (present/absent)	70/17	16/4	0.963

AFP, alpha-fetoprotein; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; UICC, International Union against Cancer.

HCC recurrence was observed in 86 (80.4%) of the 107 patients. In the majority (97.7%) of patients with recurrence, the recurrence was observed in the residual liver. Among these 86 patients, 43 (50%) and seven (8.1%) developed multinodular and extrahepatic recurrence respectively. Although no correlations were observed between these recurrence patterns and GPC3 expression, GPC3 can only be used as an indicator of poor overall survival in HCC patients.

Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of the GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period (Fig. 2b). The mean and median follow-up periods were 3.7 ± 2.1 and 3.7 years respectively. The 1-, 3-, and 5-year survival rates of the GPC3-positive HCC group were 84.4, 62.5, and 32.8% respectively. With regard to the tumor grade of HCC, 9 (11.3%) of the 80 patients with well-differentiated tumors showed significantly better prognosis without any record of deaths, compared with 71 (88.7%) patients with moderately and poorly differentiated HCC (Fig. 2c).

Further, among the 71 initial treatment patients who underwent hepatectomy and were found on histopathological examination to have moderately and poorly differentiated HCC, there were no deaths during the follow-up period in the GPC3-negative HCC group (Fig. 2d). The mean and median follow-up periods were 3.6 ± 2.0 and 3.6 years respectively.

Univariate and multivariate analyses to identify the prognostic variables in HCC patients. To identify the variables of potential prognostic significance in all the patients with HCC, univariate analysis of each variable was carried out in relation to the survival time. The difference in the prognosis was assessed by examining the relative hazard and P-value for each variable. The relative importance of each variable was then determined by multivariate Cox proportional hazards model analysis. Univariate analysis with stepwise inclusion of variables in the model revealed that the significant prognostic factors were GPC3 expression status, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, indocyanine green-retention at 15 min (ICG-R15), serum protein induced by vitamin K absence II (PIVKA-II), tumor occurence, number of tumors, resection volume, pathological bile duct involvement, and pathological intrahepatic metastasis (Table 2). However, the multivariate analysis identified only GPC3 expression (P = 0.034), intrahepatic metastasis (P = 0.027), and multiple tumors (P = 0.006) as the independent prognostic factors related to overall survival (Table 2).

Discussion

In this study, we characterized the association between the expression level of GPC3 and the malignancy grade, and the prognostic value of GPC3 in HCC. Higher levels of GPC3 expression were observed in moderately or poorly differentiated tumor cells, which was in agreement with previous reports.[19] Our contingency table analysis showed that the GPC3 expression level was correlated with the tumor differentiation level. In addition, Kaplan–Meier survival analysis revealed that GPC3 expression was significantly linked to a poor prognosis after surgical resection in HCC patients. Moreover, univariate analysis indicated that GPC3 expression is associated with an increased risk of death from HCC, and this risk factor could still be extracted in a multivariate setting. On the other hand, multivariate analysis did not identify the tumor differentiation level as an independent predictive factor of the prognosis. Among the 80 HCC patients who underwent initial surgical treatment, the GPC3-negative patients showed better prognosis than the GPC3-positive patients. Patients with well-differentiated HCC also showed a better prognosis than those with moderately and poorly differentiated HCC. Furthermore, we confirmed that among the previously treated subjects, the GPC3-negative group had a better prognosis than the GPC3-positive group with moderately and poorly differentiated HCC tumors.