detection of metastatic disease (Fig. 5B). When injected with more than 2×10^8 PFU of OBP-401, mice often showed GFP fluorescence in normal tissues such as liver, lung, spleen, and thoracic duct (data not shown). Theses results suggest that colorectal liver metastases can be visualized by GFP fluorescence both by portal venous and i.v. administration of OBP-401.

Selective Visualization of Orthotopic HCC by OBP-401

Five days after injection of OBP-401 (1×10^8 PFU/mouse) into the tail vein, HCC liver tumors were visualized by GFP fluorescence (Fig. 6A). Cross-sections of the liver at 4 weeks after i.v. injection of OBP-401 showed that GFP expression was in the cancer cells and not in normal cells (Fig. 6B and C). Small liver tumor nodules were also visualized by GFP fluorescence after i.v. OBP-401 administration (Fig. 6D). Thus, we showed that HCC liver tumors could be selectively visualized by GFP fluorescence after i.v. injection of OBP-401.

Many studies have shown that the majority of malignant human tumors tested express hTERT. OBP-301 and OBP-401 specifically replicate in tumors due to hTERT expression in tumors (11, 12, 17–19). In previous studies, OBP-301 and OBP-401 were administered locally, such as by intratumoral or intrapleural administration. The present report shows the systemic efficacy of OBP-301 and OBP-401 to selectively replicate in and kill and label primary and metastatic liver tumors after i.v. administration. Closely related virus constructs will be compared with OBP-301 and OBP-401 in the future.

Our laboratory pioneered the use of fluorescent proteins to visualize cancer cells *in vivo*. Cancer cells genetically labeled by fluorescent proteins have increased the possibility and sensitivity to observe progression of cancer cells in live animals (21). To evaluate antitumor efficacy of i.v. administration of OBP-301 against primary and metastatic liver tumors, we used GFP-expressing human cancer cell lines. We showed that i.v. administration of OBP-301 resulted in a significant reduction in experimental liver and pulmonary metastases in a colorectal liver metastases model and effectively inhibited tumor formation and growth in an orthotopic HCC model. OBP-401 has less but still significant cytotoxic effects compared with OBP-301 (22). In fact, a significant inhibition of tumor growth by intratumoral injection of OBP-401 was confirmed *in vivo* in our previous study (20). However, OBP-401 at the tumor-selective labeling dose used in this i.v. injection study could not inhibit tumor growth effectively.

The imaging strategy using OBP-401 has a potential of being available in humans as a navigation system in the surgical treatment of malignancy. During surgery, tumors that would be difficult to detect by direct visual detection could be positively identified with GFP fluorescence using a handheld excitation light and appropriate filter goggles as we have shown previously in mice (23–25). Employment of a fluorescence surgical microscope would enable visualization of the GFP-expressing microscopic leading edge of the tumor and allow accurate resection with sufficient margins.

As for toxicity of OBP-301 and OBP-401, only when injected with 5×10^8 PFU OBP-301 for the first time, a few mice showed lethargy but fully recovered within 1 h. None of the mice treated with OBP-301 or OBP-401 at the doses used in this study showed significant adverse effects during the observation period or histopathologic changes in the liver at the time of sacrifice. In the near future, the safety of OBP-301 will be confirmed in a phase I clinical trial, which is currently under way (26).

Our studies suggest the clinical potential of OBP-301 and OBP-401.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Expert Opinion

1. Introduction
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3. Telomerase-specific oncolytic adenovirus for SCCHN diagnostics
4. Clinical application of Telomelysin
5. Expert opinion

Telomerase-specific virotherapy for human squamous cell carcinoma

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Background: Replication-selective tumor-specific viruses present a novel approach for treatment of neoplastic disease. They are designed to induce lysis after propagation within the tumor. Human telomerase is active in over 85% of primary cancers and its activity correlates closely with human telomerase reverse transcriptase (hTERT) expression. *Objectives*: Oncolytic viruses, Telomelysin and TelomeScan, that combine the specificity of hTERT promoter-based expression systems with the lytic efficacy of replicative viruses were developed. The goal was to confirm the efficacy of the viruses for human squamous cell carcinoma. *Results/conclusion*: Squamous cell carcinoma of the head and neck (SCCHN) is characterized by locoregional spread, and is clinically accessible, making it an attractive target for intratumoral virotherapy. The viruses replicated efficiently and induced killing in a panel of human cancer cell lines including SCCHN cells *in vitro* and *in vivo*. These results illustrate the potential of telomerase-specific oncolytic viruses for treatment of human SCCHN.

Keywords: adenovirus, GFP, hTERT, imaging, SCCHN, telomerase

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1. Introduction

Oncolytic virotherapy has become a reality on the basis of the safety of many types of viral vectors used for human gene therapy. Viruses are the simplest form of life, carry genetic materials and are capable of entering host cells efficiently. Because of this property, many viruses have been adapted as gene transfer vectors [1-7]. Adenoviruses have been studied extensively and are well characterized. Adenoviruses are large, double-stranded DNA viruses with tropism for many human tissues such as bronchial epithelia, hepatocytes and neurons. Furthermore, they are capable of transducing nonreplicating cells and can be grown to high titers *in vitro*, which allows for their potential use clinically. High titers of replication-defective adenoviruses can be produced and have been successfully used in eukaryotic gene expression [1,8,9]. Numerous studies using *in vitro* and animal models have tested a wide variety of adenoviral gene therapy agents and reported potential beneficial effects for different target diseases, and their tolerability and safety [10-13].

Gene and vector-based therapies for cancer encompass a wide range of treatment types that all use genetic material to modify cancer cells and/or surrounding tissues to make them exhibit antitumor properties. One of the most common approaches to emerge from the concept of gene therapy is the introduction of foreign therapeutic genes into target cells. A number of genes of interest with different functions such as tumor suppressor genes [14,15], proapoptotic genes [16,17], suicide genes that cause cellular death with prodrugs [13,18], and genes that inhibit angiogenesis [19] have been proposed for this type of therapy. In fact, the author's group and others have completed clinical trials of a replication-deficient adenoviral

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vector that delivers normally functioning *p53* tumor suppressor gene to cancer cells (Ad5CMV-p53, Advexin). It has been reported that multiple courses of intratumoral injection of Ad5CMV-p53 are feasible and well tolerated in patients with advanced head and neck squamous cell carcinoma and non-small cell lung cancers and appear to provide clinical benefits [20-24]. Another rapidly growing area of gene therapy for cancer is the use of oncolytic vectors for selective tumor cell destruction. Since viruses infect cells and then induce cell lysis through their propagation, they can be used as anticancer agents by genetic engineering that causes them to replicate selectively in cancer cells while remaining innocuous to normal tissues [25]. Clinical trials of intratumoral injection of Onyx-015, which is an adenovirus with the E1B 55-kDa gene deleted, engineered to selectively replicate in and lyse p53-deficient cancer cells [26], alone or in combination with cisplatin/5-fluorouracil have been conducted in patients with recurrent head and neck cancer [27,28]; however, the study afterwards has clarified that the capacity of Onyx-015 to replicate independently of the cell cycle does not correlate with the status of p53 but is determined by yet unidentified mechanisms [29].

The optimal treatment of human cancer requires improvement of the therapeutic ratio to increase the cytotoxic efficacy on tumor cells and decrease that on normal cells. This may not be an easy task because the majority of normal cells surrounding tumors are sensitive to cytotoxic agents. Thus, to establish reliable therapeutic strategies for human cancer, it is important to seek genetic and epigenetic targets present only in cancer cells. One of the targeting strategies has involved the use of tissue-specific promoters to restrict gene expression or viral replication in specific tissues. Telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes, and contains three components: the RNA subunit (known as hTR, hTER, or hTERC) [30], the telomerase-associated protein (hTEP1) [31], and the catalytic subunit (hTERT, human telomerase reverse transcriptase) [32,33]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [34] and, therefore, represent the minimal catalytic core of telomerase in humans [35]. However, while hTR is widely expressed in embryonic and somatic tissues, hTERT is tightly regulated and is not detectable in most somatic cells. The hTERT proximal promoter can be used as a molecular switch for the selective expression of target genes in tumor cells, since almost all advanced human cancer cells express telomerase while most normal cells do not [36,37].

An estimated 500,000 patients worldwide are diagnosed with squamous cell carcinoma of the head and neck (SCCHN) annually [38]. This aggressive epithelial malignancy is associated with a high mortality rate and severe morbidity among the long-term survivors [39]. Current treatment strategies for advanced SCCHN include surgical resection, radiation and cytotoxic chemotherapy. Although a combination of these modalities can improve survival, most patients eventually experience disease progression that leads to death; disease progression is often the result of intrinsic or acquired resistance to treatment [40,41]. A lack of specificity for tumor cells is the primary limitation of radiotherapy and chemotherapy. To improve the therapeutic index, there is a need for anticancer agents that selectively target only tumor cells and spare normal cells. This review looks at recent developments in this rapidly evolving field, cancer therapeutic and cancer diagnostic approaches using the hTERT promoter, and highlights some very promising advances for the treatment of human SCCHN.

2. Telomerase-specific oncolytic virotherapy for human SCCHN

2.1 hTERT promoter-driven oncolytic adenovirus

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*. 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 [42]. 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 [43-46]. Furthermore, Kuppuswamy *et al.* have recently developed a novel oncolytic adenovirus (VRX-011), in which the replication of the vector targets cancer cells by replacing adenovirus E4 promoter with the hTERT promoter [47]. VRX-011 could also overexpress the adenovirus death protein (ADP) (also known as E3-11.6K), which is required for efficient cell lysis and release of virions from cells at late stages of infection.

The adenovirus *E1B* gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promotes 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.* [48] have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α-fetoprotein (AFP) promoter with the use of internal ribosome entry sites (IRES) significantly improved the specificity and the therapeutic index in hepatocellular carcinoma cells. Based on the above information, Telomelysin (OBP-301) was developed, in which the tumor-specific hTERT promoter regulates both the *E1A* and *E1B* genes (Figure 1). Telomelysin is expected to control

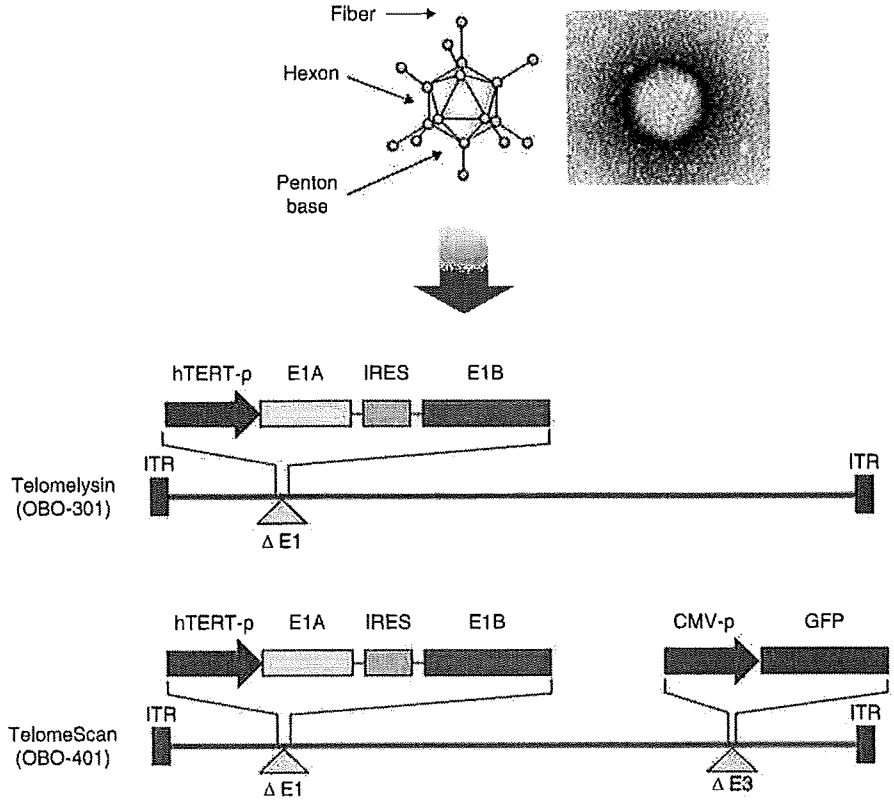

Figure 1. Structures of telomerase-specific oncolytic adenoviruses. Telomelysin (OBP-301), in which the humantelomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES). TelomeScan (OBP-401) is a telomerase-specific replication-competent adenovirus variant, in which the green fluorescent protein (*GFP*) gene is inserted under the control of the cytomegalovirus (CMV) promoter into the E3 region for monitoring viral replication. Upper panel, schematic representation depicting major structural components of Telomelysin (hexon, penton base and fiber) and transmission electron microscopic image.

viral replication more stringently, thereby providing better therapeutic effects in tumor cells as well as attenuated toxicity in normal tissues [49].

2.2 *In vitro* cytopathic efficacy of Telomelysin in human SCCHN cell lines

The majority of human cancer cells including human SCCHN cells acquire immortality and unregulated proliferation by expression of hTERT [42] and, therefore theoretically, hTERT-specific Telomelysin can possess a broad-spectrum antineoplastic activity against a variety of human tumors [49,50]. Indeed, although the levels of expression varied widely, it was confirmed by using a real-time RT-PCR method that all SCCHN cell lines expressed detectable levels of *hTERT* mRNA, whereas human fibroblast cells were negative for *hTERT* expression. The author's group also examined the expression levels of coxsackievirus and adenovirus receptor (CAR) on the cell surface of each type of cell by flow cytometric analysis. Appreciable amounts of CAR expression were detected on human SCCHN cells. Thus, Telomelysin infection could efficiently induce cell death in a variety of human SCCHN cell lines such as SAS-L, SCC-4, SCC-9, HSC-2, HSC-3 and HSC-4 in a dose-dependent manner; the sensitivity, however, varied among different cell lines [51]. Telomelysin induced selective E1A and E1B expression in these SCCNH cells, which resulted in viral replication at 4 logs by 24 h after infection; on the other hand, Telomelysin replication was attenuated up to 2 logs in cultured normal cells. These data clearly demonstrate that Telomelysin exhibits desirable features for use as an oncolytic therapeutic agent for human SCCHN.

2.3 *In vivo* antitumor effect of Telomelysin in human SCCHN xenografts

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 [49,50]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose Telomelysin, indicating that Telomelysin induced intratumoral necrosis due to direct lysis of tumor cells by virus replication *in vivo* [52,53].

To further explore the *in vivo* antitumor effects of telomerase-specific virotherapy for SCCHN, we used an orthotopic nude mouse model of human tongue squamous cell carcinoma. An orthotopic nude mouse model to investigate the cellular and molecular mechanisms of metastasis in human neoplasia was first described by Fidler *et al.* [54,55] and Killion *et al.* [56]. The orthotopic implantation of tumor cells restores the correct tumor–host interactions, which do not occur when tumors are implanted in ectopic subcutaneous sites [54]. In our preliminary experiments, we inoculated tumor cells into the tongue of BALB/c *nu/nu* mice and confirmed the formation of tumors with a diameter of 3 – 5 mm after 7 days and the development of metastases in neck lymph nodes after 35 days. Intratumoral injection of Telomelysin significantly shrunk the tongue tumor volumes, which in turn increased the body weight of mice by enabling oral ingestion. Since the body weight loss due to a feeding problem in this orthotopic SCCHN model resembles the disease progression in SCCHN patients, the finding that Telomelysin increased the body weight of mice suggests that telomerase-specific virotherapy could potentially improve the quality of life in advanced SCCHN patients (Figure 2).

3. Telomerase-specific oncolytic adenovirus for SCCHN diagnostics

3.1 hTERT promoter-driven *GFP*-expressing oncolytic adenovir

The green fluorescent protein (GFP), which was originally obtained from the jellyfish *Aequorea victoria*, is an attractive molecular marker for imaging of live tissues because of the relatively non-invasive nature of the fluorescence [57]. To label target tumor cells efficiently and uniformly with green fluorescence, we modified Telomelysin to contain the *GFP* gene driven by the cytomegalovirus (CMV) promoter in the E3 deleted region. The resultant adenovirus was termed TelomeScan or OBP-401 (Figure 1) [58,59]. Similar to Telomelysin, TelomeScan replicated in human cancer cell lines and coordinately induced GFP expression; TelomeScan replication, however, was attenuated in normal human fibroblasts without GFP expression.

Human SCCHN cells also expressed bright GFP fluorescence after TelomeScan infection. The fluorescence intensity gradually increased in a dose-dependent manner, followed by rapid cell death due to the cytopathic effect of TelomeScan, as evidenced by the presence of floating, highly light-refractive cells under phase-contrast photomicrographs. We also quantified GFP expression in human SCCHN cells following TelomeScan infection by using a fluorescence plate reader. Relative expression levels of GFP gradually increased in a dose-dependent manner. Moreover, we found an apparent inverse correlation between relative GFP expression at 72 h after TelomeScan infection and cell killing effects of Telomelysin in monolayer cultures (defined as ID_{50}) in various human cancer cell lines including SCCHN cell lines, indicating that the outcome of Telomelysin treatment could be predicted by measuring GFP expression following TelomeScan infection. For example, when the biopsy tissue samples of the tumor are exposed to TelomeScan for a certain amount of time *ex vivo*, the levels of GFP expression may be of value as a positive predictive marker for the outcome of Telomelysin virotherapy (Figure 2).

3.2 *In vivo* imaging of SCCHN micrometastasis with Telomescan

Improvements in methods of external imaging such as computed tomography (CT), MRI and ultrasound techniques have increased the sensitivity for visualizing tumors and metastases in the body [60]. Positron emission tomography (PET) using the glucose analogue ^{18}F-2-deoxy-D-glucose (FDG), was the first molecular imaging technique to be widely applied for cancer imaging in clinical settings [61]. Although FDG-PET has high detection sensitivity, it has some limitations such as difficulty in distinguishing between proliferating tumor cells and inflammation, and its unsuitability for real-time detection of tumor tissues. Therefore, tumor-specific imaging is of considerable value in the treatment of human cancer because it can define 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.

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. Indeed, SCCHN patients with metastases to regional lymph nodes have a poorer prognosis than patients without nodal metastases [62]. Therefore, the utility of TelomeScan, which 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. We have previously demonstrated that TelomeScan could be delivered into human tumor cells in regional lymph nodes and replicate with selective GFP fluorescence after injection into the primary tumor in an orthotopic rectal tumor model [63]. In the orthotopic SCCHN model, TelomeScan also spread into the neck lymph nodes after injection into the primary tongue tumor and selectively replicated in metastatic nodules. Although the virus replication can not catch up in tumors with an extremely rapid progress, leading to the incomplete tumor eradication, these results suggest

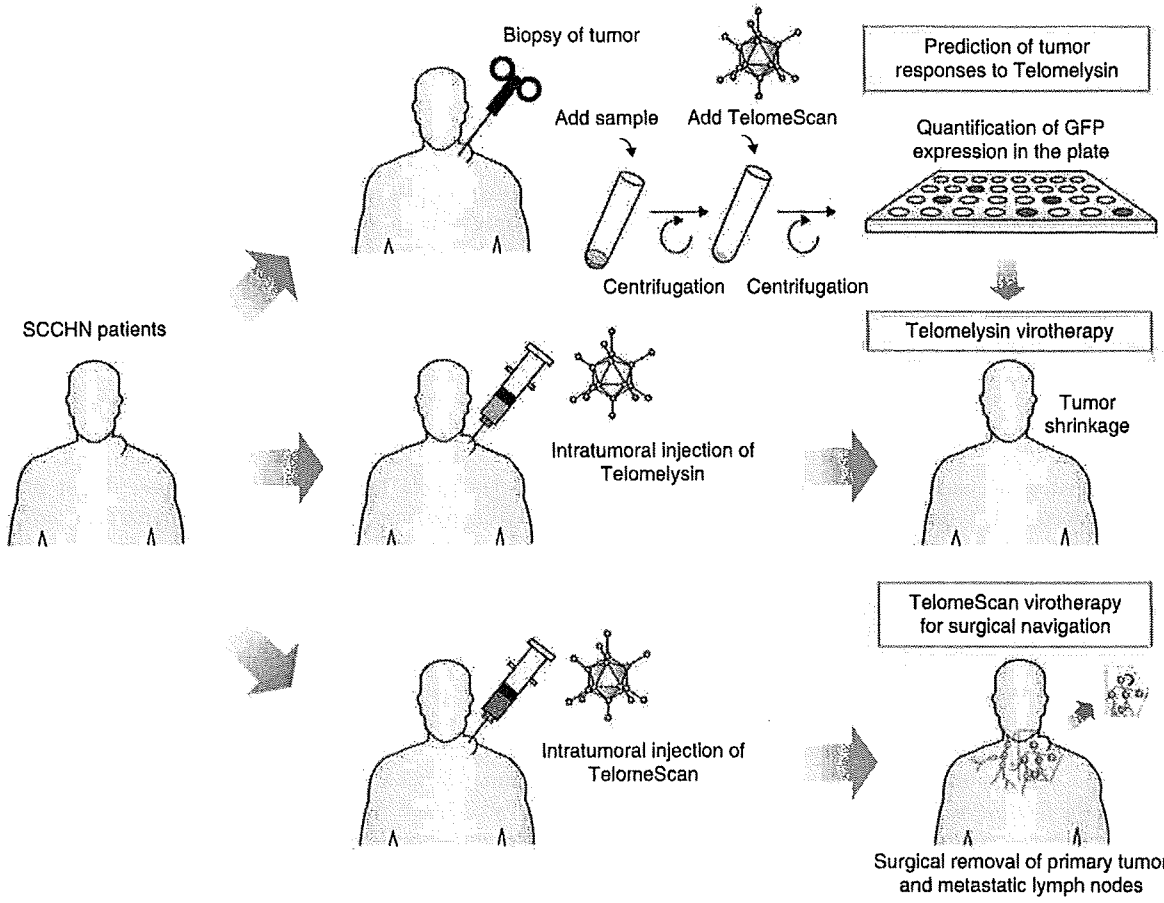

Figure 2. A schematic representation of diagnostic and therapeutic approaches using telomerase-specific oncolytic adenoviruses for human squamous cell carcinoma of the head and neck (SCCHN) patients. Top row: The outcome of Telomelysin treatment can be predicted by measuring GFP expression following TelomeScan infection. When the biopsy tissue samples of the tumor are exposed to TelomeScan *ex vivo*, the levels of GFP expression may be of value as a positive predictive marker for the outcome of Telomelysin virotherapy. Middle row: Intratumoral injection of Telomelysin may reduce the tumor volumes, which could potentially improve the quality of life in advanced SCCHN patients. Bottom row: TelomeScan can spread into the neck lymph nodes after injection into the primary tumors and selectively express GFP fluorescence in metastatic nodules. Surgeons may be able to excise primary tumors as well as metastatic lymph nodes precisely with appropriate margins by using this novel surgical navigation system with TelomeScan.

that surgeons may be able to excise primary tumors as well as metastatic lymph nodes precisely with appropriate margins by using this novel surgical navigation system with TelomeScan (Figure 2).

Administration of TelomeScan offers an additional advantage in cancer therapy. TelomeScan, like Telomelysin, is an oncolytic virus, and selectively kills human tumor cells by viral replication; the process of cell death by TelomeScan, however, is relatively slow compared with apoptosis-inducing chemotherapeutic drugs, because the virus needs time for replication. Therefore, tumor cells infected with TelomeScan express GFP fluorescence, followed by loss of viability, allowing the timing of detection. Thus, TelomeScan 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.

4. 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 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 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, three-cohort dose-escalation study [52]. The trial commenced following approval by the FDA in October, 2006. The study to assess the safety, tolerability, and feasibility of intratumoral injection of the agent in patients with advanced solid cancer has almost been completed. The author and colleagues also analyzed the humoral immune response to Telomelysin, and obatined tissue biopsies to evaluate the pharmacokinetics and pharmacodynamics of Telomelysin in the injected tumor. The therapeutic responses were 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 have failed to respond to conventional therapies such as primary external beam radiation or systemic chemotherapy. All patients had a disease that is measurable and accessible to direct injection of Telomelysin. The doses of Telomelysin were escalated from low to high virus particles (VP) in one log increments. Patients were treated with a single dose intratumoral injection of Telomelysin and then monitored over one month.

Although the final report is not available yet, the data on pharmacokinetics and biodistribution of Telomelysin may be of interest. 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 [64,65], 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 organs and 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 an antitumor effect. The preclinical study, however, showed that multiple injections of Telomelysin resulted in a profound inhibition of tumor growth in xenograft models [49,50,59]. Thus, we also evaluated the feasibility of the multi-cycle treatment with Telomelysin.

5. Expert opinion

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, many ethical and technical hurdles remain to be tackled and must be solved before virotherapy, including virus-mediated gene therapy, ever reaches 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 resulting from viral replication, and is a promising therapeutic alternative to replication-deficient gene therapy vectors. Several independent studies that used different regions of the hTERT promoter and different sites of the adenoviral genome responsible for viral replication, have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effects in a variety of human tumor cells including SCCHN cells [43-45,49-51]. 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.

SCCHN accounts for 5% of newly diagnosed adult cancers in the United States and 8% of cancers worldwide [66]. Most patients are treated with various combinations of surgery, radiotherapy and systemic agents [67]. Despite major advances in the treatment of locoregionally advanced SCCHN such as the introduction of novel chemotherapy regimens, treatment fails in about half of the patients [68]. The median survival of patients with recurrent or metastatic SCCHN who undergo chemotherapy is 6 – 9 months [69]. A considerable number of patients with SCCHN need additional treatment as the disease progresses. Targeted therapies such as the anti-EGFR monoclonal antibody cetuximab and other small-molecule EGFR-tyrosine kinase inhibitors have been developed for SCCHN. Although a Phase III trial demonstrated a survival benefit with cetuximab and standard platinum-based therapy in SCCHN patients [70], some patients are exquisitely sensitive to these drugs and can develop particular and severe toxicities [71]. An interim analysis of a Phase I study of Telomelysin for histologically proven non-resectable solid tumors including SCCHN patients indicates that Telomelysin virotherapy is well-tolerated without any severe adverse events [52], suggesting that Telomelysin may be much more potent than other targeted therapies for human SCCHN in terms of specificity, efficacy and toxicity.

Although Telomelysin showed a broad and profound antitumor effect in human SCCHN cells, one weakness of Telomelysin is that virus infection efficiency depends on CAR expression, which may not be highly expressed on the cell surface of some types of human SCCHN cells. Thus, tumors that lost CAR expression might 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. 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 [50]. Telomelysin-RGD had an apparent oncolytic effect on human cancer cell lines with extremely 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 range 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 [72].

Possible future directions for Telomelysin include combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy and new modalities such as antiangiogenic therapy. Since clinical activities observed with intratumoral injection of Telomelysin suggest that even partial elimination of the SCCHN tumor could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human SCCHN. The combination of systemic chemotherapy and local injection of Telomelysin has been previously shown to be effective [58,59]. 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 [73]. Therefore, Telomelysin can be effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that induces specific cytotoxic T-lymphocytes against the remaining antigen-bearing tumor cells. We also confirmed that Telomelysin seems to have antiangiogenic properties through the stimulation of host immune cells to produce endogenous antiangiogenic factors such as IFN-γ and interleukin 12. Peri- or postoperative administration of Telomelysin may be valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence or regrowth of SCCHN tumors.

The field of telomerase-specific gene- and vector-based therapies is progressing considerably and is rapidly gaining medical and scientific acceptance. Although many technical and conceptual problems remain to be solved, ongoing and future clinical studies will no doubt continue to provide important clues that may allow substantial progress in the treatment of human SCCHN.

Declaration of interest

The author is a Chief Scientific Officer of Oncolys BioPharm, Inc.

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

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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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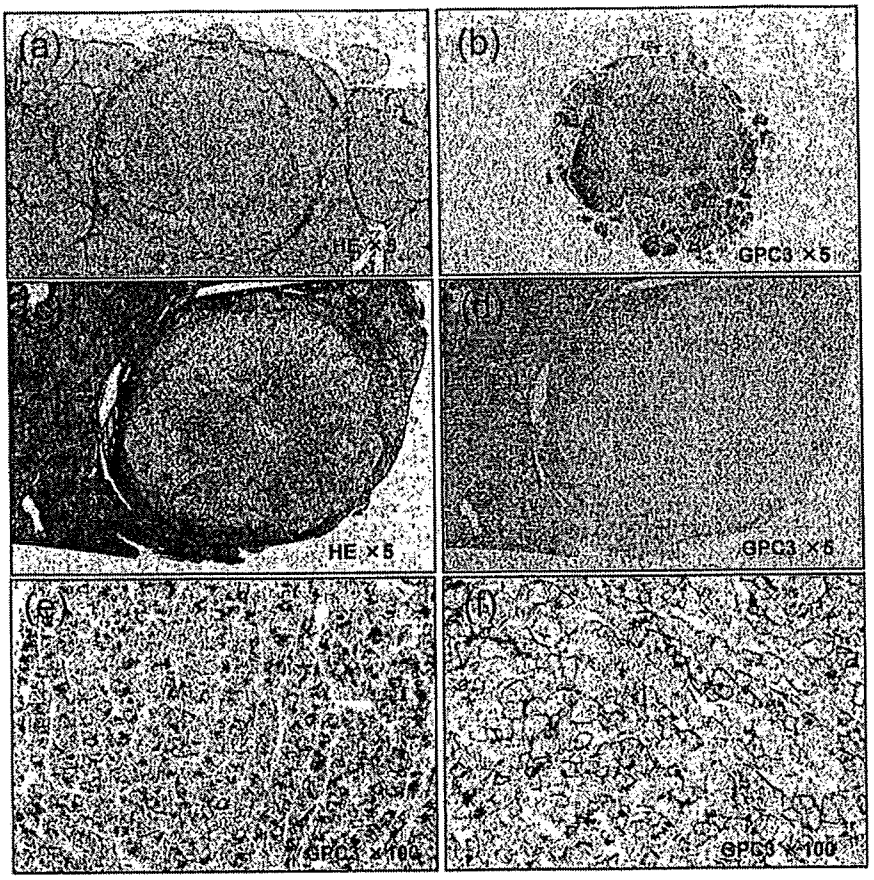

Fig. 1. Glypican (GPC)-3 expression and localization is hepatocellular carcinoma (HCC)-specific. (a,c) Microscopic view of a HE-stained sections of resected HCC. (b,d) HCC sections were stained for GPC3 expression with anti-GPC3 monoclonal antibody. (e) HCC displays prominent bile-canalicular immunostaining.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

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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.

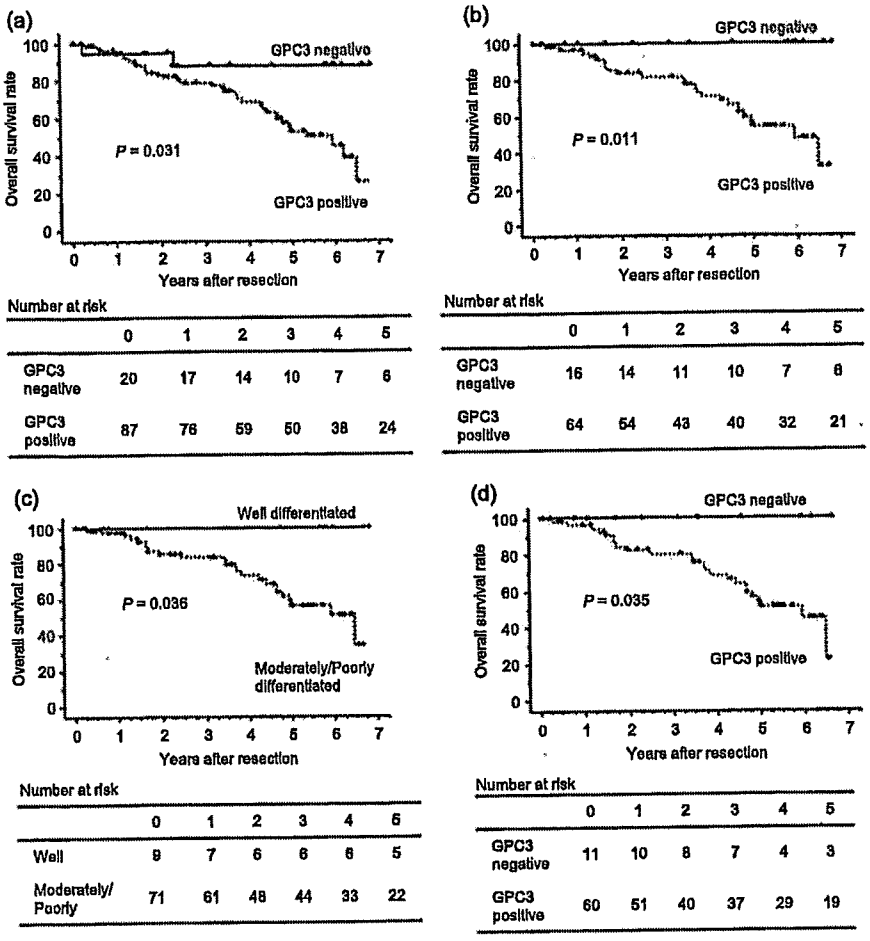

Fig. 2. Overall survival curves for the 107 hepatocellular carcinoma (HCC) patients stratified into those with glypican (GPC)-3-positive and GPC3-negative HCC. (a) Overall survival of patients with GPC3-positive HCC was shorter than those with GPC3-negative HCC ($P = 0.031$). (b) Overall survival curves in 80 of 107 HCC patients with initial treatment who underwent hepatectomy with positive and negative GPC3 expression. Patients with GPC3-positive HCC had a lower 5-year survival than those with GPC3-negative HCC ($P = 0.011$). (c) Overall survival curves in the 71 HCC patients with initial hepatectomy who exhibited well- and moderately and poorly differentiated HCC on histopathological examination. The 5-year survival rate was lower in the moderately and poorly differentiated GPC3-positive HCC than in the corresponding GPC3-negative HCC ($P = 0.036$). (d) Overall survival curves in the 71 initial treatment patients who underwent hepatectomy and exhibited moderately and poorly differentiated HCC on pathological examination with positive and negative GPC3 expression. The 5-year survival rate was lower in the GPC3-positive HCC patients than in the GPC3-negative HCC patients ($P = 0.035$).

Table 2. Prognostic factors for overall survival by univariate and multivariate analyses

Variable	No. patients	Univariate analysis		Multivariate analysis		
		5-year survival rate (%)	*P*-value	RR	95% CI	*P*-value
Age (years) (≥65/<65)	51/56	65.8/53.4	0.531			
Sex (male vs female)	85/22	56.1/72.7	0.403			
HBsAg (positive vs negative)	29/78	51.0/62.3	0.011	1.14	0.31–4.16	0.844
HCV (positive vs negative)	62/45	66.7/46.4	0.004	2.41	0.75–7.69	0.138
ICG R15 (%) (≥15 vs <15)	50/57	70.3/46.8	0.047	0.69	0.31–1.54	0.362
AFP (ng/mL) (≥50 vs <50)	45/62	49.1/65.1	0.132			
PIVKA-II (mAU/mL) (≥700 vs <700)	30/77	35.0/65.6	0.016	1.91	0.730–5.02	0.188
Tumor occurring (first vs recurrence)	80/27	62.8/50.2	0.019	1.83	0.78–4.31	0.167
No. tumors (solitary vs multiple)	75/32	65.7/42.7	0.009	3.53	1.41–8.00	0.006
Resection (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	29/78	36.5/67.1	0.005	1.71	0.52–5.60	0.374
Operation time (min) (>300 vs ≤300)	49/58	43.9/72.3	0.053			
Intraoperative blood loss (mL) (≥1300 vs <1300)	42/65	42.3/68.8	0.097			
Perioperative transfusion (present vs absent)	54/53	49.6/66.5	0.599			
Tumor size (mm) (>50 vs ≤50)	38/69	51.5/62.5	0.154			
Histological differentiation (well vs moderately and poorly)	12/95	77.8/56.4	0.102			
pStage (I vs II/III)	41/66	64.2/56.5	0.071			
Portal vein involvement (present vs absent)	47/60	64.9/58.5	0.369			
Hepatic vein involvement (present vs absent)	10/97	44.4/60.5	0.060			
Bile duct involvement (present vs absent)	12/95	20.0/62.7	0.004	0.94	0.31–2.85	0.912
Intrahepatic metastasis (present vs absent)	24/83	29.0/66.6	0.001	3.57	1.13–10.50	0.027
Non-cancerous lesion (cirrhosis vs non-cirrhosis)	40/67	53.6/61.9	0.232			
GPC3 staining (positive vs negative)	87/20	54.5/87.7	0.025	5.26	1.13–24.39	0.034

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

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In this study, the patients who were HCV positive, had higher ICG-R15 values, or portal vein involvement showed longer survival times, especially the patients who were HCV-positive or had higher ICG-R15 values, showed statistical significance in the univariate analysis. However, there was no statistical significance in these variables in the multivariate analysis. The reasons for these contradictive results in the univariate analysis are unclear.

In contrast, subgroup analysis did not reveal any significant difference in the disease-free survival rate between the GPC3-positive and GPC3-negative HCC patients (data not shown). The rate of recurrence in patients after surgery was 63.8% within the first 2 years after surgery among the previously treated patients in this study. Tumor recurrence in the GPC3-positive HCC patients occurred earlier than that in the GPC3-negative HCC patients until 9.7 months after the surgery among the patients who had received previous treatment. Two mechanisms of postoperative recurrence of HCC have been suggested: one is intrahepatic metastasis in the residual liver in a metachronous manner, and the other is multicentric hepatocarcinogenesis based on chronic hepatitis.[20–23] Some authors have suggested that early recurrence arises most often from intrahepatic metastases, whereas late recurrence is more likely to be multicentric in origin. Poon *et al.* and Portolani *et al.* reported that tumor factors like neoplastic vascular infiltration, but not host factors, were linked to early recurrence, whereas the risk of late recurrence was dependent on the underlying liver status.[21,22] These results indicate that GPC3 expression may indicate a high risk of intrahepatic recurrence.

Most of the GPC3 expression patterns in HCC cells showed the cytoplasmic pattern. There was no case that showed only the membrane pattern. Almost half of the HCC cases showed the mixed pattern (cytoplasm and membrane) and the other half showed only the cytoplasmic pattern.

There was no statistical significance between the mixed pattern (cytoplasm and membrane) and cytoplasmic pattern ($P = 0.297$) in Kaplan–Meier survival analysis. The functional difference between cytoplasmic GPC3 and membrane GPC3 is unknown, so further investigations are needed to clarify whether the different localization of staining has a different significance.

In addition to the investigation of its role as a prognostic indicator, a phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC is now underway; GPC3 is an ideal target for this therapy because it is more effective in patients with increased expression of GPC3, which is frequently observed in the later stages of HCC, as shown in the present study. The poor prognosis of patients with GPC3-positive HCC also prompted us to develop a strategy of anticancer immunotherapy,[24,25] that is, we may expect the effect of hepatocarcinogenesis prevention after surgery in patients with GPC3-positive HCC.

In summary, our study evaluated the prognostic significance of GPC3 expression at the protein level in clinical tissue specimens of HCC. The overall survival rate was significantly poorer in patients with elevated GPC3 expression in the tumor than in those with lower levels of GPC3 expression. Further functional characterization of GPC3 may be expected to lead to a better understanding of the molecular mechanisms underlying the development and progression of HCC.

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Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis

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Abstract. Glypican-3 (GPC3) is one of carcinoembryonic antigens known to be overexpressed in hepatocellular carcinoma (HCC). It has been suggested that GPC3 may be related to the development of HCC in a background of chronic hepatitis (CH) and liver cirrhosis (LC). Therefore, in an attempt to establish an early diagnostic marker of HCC, we quantified the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients. We selected CH and LC patients who were HCV-RNA (+) or HBs antigen (+) within 6 months prior to the study and had no HCC nodules as detected by imaging. A total of 56 patients with CH and LC, and 45 patients with HLA-A24$^+$ or HLA-A2$^+$ were enrolled for this investigation. After isolation of mononuclear cells from each patient's peripheral blood specimens, we performed ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides. In the ELISPOT assay, GPC3-specific CTLs were detected in 10 of the 45 CH and LC cases (22%). In addition, the plasma titers of anti-GPC3 IgG were increased in the CH and LC patients as compared with those in healthy donors. GPC3-specific CTLs were found to be present not only in patients with HCC, but also in patients with CH and LC. This suggests the possibility of GPC3-specific CTLs serving as a marker for the early diagnosis of imaging-invisible HCC.

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Abbreviations: GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Key words: glypican-3, CTL, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

Introduction

The prevalence of hepatocellular carcinoma (HCC) is increasing rapidly in both Asian and Western countries. It is clear that patients with hepatitis B- or C-associated liver cirrhosis are at a higher risk of developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at a higher risk of recurrence (2). Furthermore, the liver function of these patients is often very poor, which restricts further treatment options for recurrence. As a result, the prognosis of HCC remains poor, and the development of new therapies for the prevention of cancer development and recurrence, that is, adjuvant therapy, is urgently needed.

Glypican-3 (GPC3) has been reported to be overexpressed in most types of HCC (3-10) and melanoma in humans (6,8,9). GPC3 belongs to the six-member family of glypicans in mammals (11). GPC3 is a heparan sulfate proteoglycan that is bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor. GPC3 has been shown to regulate the signaling mediated by Wnts (12,13), Hedgehogs (14), fibroblast growth factors (15,16) and bone morphogenetic proteins (15,17). These signaling pathways are only partially dependent on the heparan sulfate chains (11,16,18). However, whether GPC3 plays an oncogenic role in HCC is still controversial.

We recently identified both HLA-A24 (A*2402) and H-2K^d-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A2 (A*0201)-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We previously reported a preclinical study conducted in a mouse model with a view to designing an optimal schedule for clinical trials of a GPC3-derived peptide vaccine (20). We predicted that overexpression of GPC3 in HCC is related to the development of HCC in a background of chronic hepatitis (CH) and/or liver cirrhosis (LC). Towards establishing the possibility of early diagnosis of imaging-invisible HCC and vaccine therapy, we determined the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients.

Materials and methods

Patients, blood samples and cell lines. Blood samples from patients with CH and LC were collected during routine diagnostic procedures after obtaining their written consent at the Tokyo Rosai Hospital between October 2006 and October 2007. CH and LC patients who were confirmed to be HCV-RNA(+) or HBs antigen(+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC, and no evidence of HCC on ultrasonography, CT (computed tomography) or MRI (magnetic resonance imaging) conducted prior to the registration.

Human liver cancer cell lines SK-Hep-1/GPC3, HepG2 and K562 were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS. SK-Hep-1/GPC3 has been described previously (19). HepG2 endogenously expressing GPC3 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). HLA-class I deficient K562 was obtained from Kumamoto University. The origins and HLA genotypes of these cell lines have been described in previous reports (21,22).

Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay. We isolated peripheral blood mononuclear cells (PBMCs) from the heparinized blood of HLA-A2$^+$ and/or HLA-A24$^+$ Japanese CH, LC or HCC patients and healthy donors by means of Ficoll-Conray density gradient centrifugation. IFN-γ production by the CTLs present in the PBMCs in the presence or absence of the GPC3 peptide was assessed by the ELISPOT assay (BD™ Bioscience, San Diego, CA), as described previously. Briefly, defrosted PBMCs (1x10^6/well) were cultured in 96-well flat-bottomed plates for the ELISPOT assay (BD Bioscience) with HLA-A2-restricted $GPC3_{44-52}$ (A2-1) (RLQPGLKWV), $GPC3_{144-152}$ (A2-3) (FVGEFFTDV), $GPC3_{155-163}$ (A2-4) (YILGSDINV) and HLA-A24-restricted $GPC3_{298-306}$ (A24-8) (EYILSLEEL) (10 μM) with 100 units/ml recombinant human IL-2 overnight *in vitro*. The negative control consisted of medium alone and the positive control included HLA-A24- or -A2-restricted cytomegalovirus. The number and area of the spots were automatically determined and subsequently analyzed with the ELISPOT system (Minerva Tech, Tokyo, Japan).

Induction of GPC3-reactive human CTLs and cytotoxic assay. We evaluated the cytotoxic activity of the CTLs that were induced with the GPC3 A2-3 peptide in the PBMCs isolated from the CH4 patient. PBMCs were isolated from HLA-A2$^+$ CH4 patient, distributed into 4 wells (3x10^5 cells/24-well), and cultured with the GPC3 A2-3 peptide. After culture for 7 and 14 days, the PBMCs cocultured with irradiated autologous monocyte-derived DCs obtained by positive selection with human CD14 Micro Beads (Miltenyi, Bergisch Gladbach, Germany) were pulsed with the GPC3 A2-3 peptide. The CD14$^+$ cells were cultured in the presence of 100 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Inc.) and 100 ng/ml of IL-4 (R&D Systems, Inc.) in RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO) containing 2% heat-inactivated autologous serum and 1% penicillin-streptomycin-glutamine (Gibco, Invitrogen, Ltd.; Paisley, Scotland, UK). After 5 days, TNFα (PEPRPTECH EC., London, UK) was added at the concentration of 20 ng/ml to induce maturation of the DCs. After 7 days, mature DCs were harvested and pulsed with 10 μM of the candidate peptides for 4 h at room temperature in RPMI. The peptide-pulsed DCs were then irradiated (3500 rads) and mixed at a ratio of 1:20 with autologous PBMCs.

These DCs were set up in 48-well culture plates; each well contained 1.5x10^4 peptide-pulsed DCs, 3x10^5 PBMCs and 5 ng/ml IL-7 (PEPRPTECH EC.) in 0.5 ml of RPMI containing 10% autologous serum. Three days after the start of the incubation, IL-2 (R&D Systems, Inc.) was added to these cultures at a final concentration of 10 U/ml. On days 7 and 14, the T cells were restimulated with the autologous DCs pulsed with the peptide.

After 21 days, the cells were recovered and analyzed for their cytotoxic activity against the target cells with the TERASCAN VPC system (Minerva Tech), as previously described (23). Briefly, SK-Hep-1/GPC3 (GPC3$^+$, A2$^+$, A24$^+$), HepG2 (GPC3$^+$, A2$^+$, A24$^+$) and K562 (HLA-class I$^-$) cells were used as the target cells and labeled with calcein-AM solution for 30 min at 37˚C. The labeled cells were washed three times and distributed into a 96-well culture plate (1x10^4 per well) and then incubated with the effector cells for 5 h. The fluorescence intensity was measured before and after 5-h culture, and the Ag-specific cytotoxic activity was calculated using the following formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100.

ELISA for the detection of anti-GPC3 IgG antibodies. Recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) was diluted in 10 x Block Ace (Dainippon Pharmaceutical, Osaka) to a final concentration of 1 μg/ml, dispensed into 96-well plates (100 μl/well) and incubated overnight at 4˚C. Then, the plates were blocked with Block Ace for 1 h at room temperature. Plasma samples from CH and LC patients and healthy controls (100 μl, 1:100 dilution) were added to each well, followed by incubation for 2 h at room temperature. After washing three times with PBS containing 0.05% Tween-20 (PBST), Peroxidase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories, Inc., W. Baltimore, USA) was reacted for 30 min. The plates were washed with PBST and developed with Stable Peroxide Substrate Buffer (Pierce, Rockford, IL) for 20 min. After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 490 nm. All plasma samples were measured in duplicate and were randomly dispensed into the plates.

Statistical analysis. The two-tailed Student's t-test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay. Unpaired Mann-Whitney U tests were used for the evaluation of the significance of differences in the data obtained by ELISA. $P<0.05$ was considered to denote significant difference.

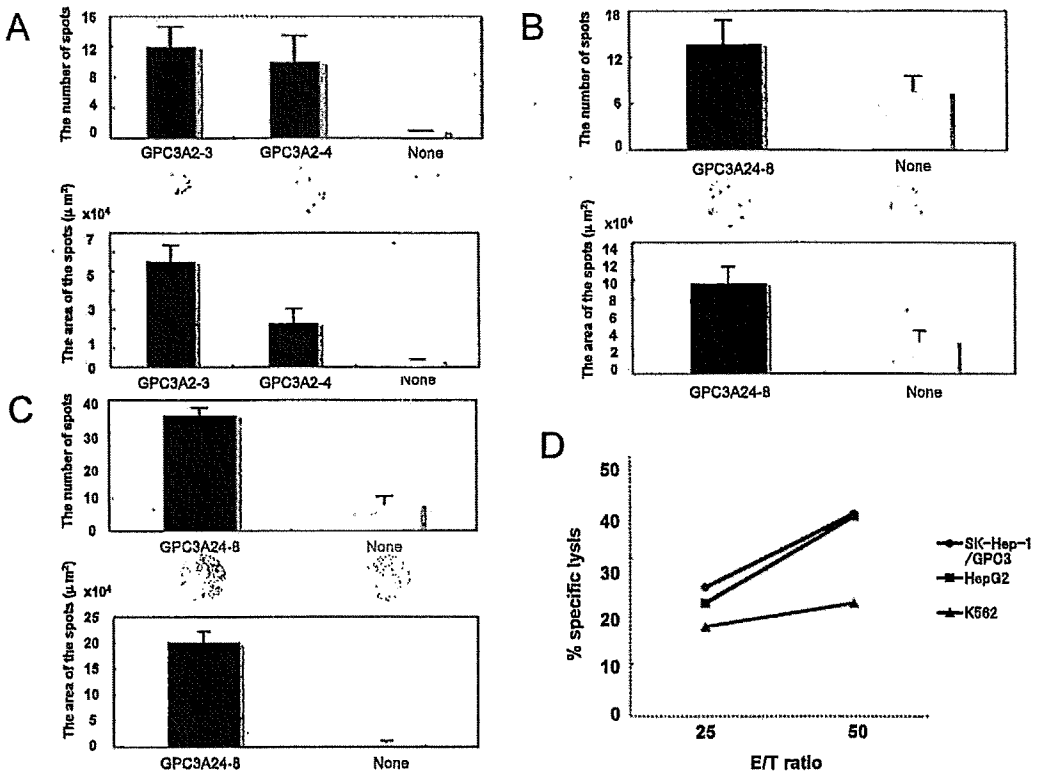

Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8+ T cells were detected in the chronic hepatitis [(A), HLA-A2+ CH4 patient; (B), HLA-A24+ CH5 patient] and liver cirrhosis [(C), HLA-A24+ LC5 patient]. IFN-γ produced by the peptide-specific T cells was measured by the IFN-γ-ELISPOT assay (middle column). The number and area of spots are shown in the upper and lower panels, respectively. Lysis of human hepatoma cell lines SK-Hep-1/GPC3 (circles) and HepG2 (squares) expressing GPC3 and HLA-A2 by GPC3-specific CTLs was observed following stimulation with the GPC3 A2-3 peptide (FVGEFFTDV) [(D), HLA-A2+ CH4 patient]. An HLA-classI- K562 human erythromyeloblastoid leukemia cell line was used as the negative control (triangles).

Results

Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH, LC and HCC patients. We evaluated the frequency of CTLs that recognized the GPC3 A2-1, A2-3, A2-4 or A24-8 peptide in the PBMCs of CH, LC and HCC patients. The CH and LC patients enrolled in this study were 34 male and 22 female patients. The average age of the patients was 64 years. HCV and HBV infection was found in 54 and 2 patients, respectively. The 56 patients were 33 CH and 23 LC cases. Mean serum α-fetoprotein (AFP) was 13.3±21.1 ng/ml (normal <20 ng/ml). In regard to the HLA genotype, 10, 22 and 13 patients, respectively, were HLA-A2+, HLA-A24+ and HLA-A2+/24+. On the other hand, there were 11 patients who were HLA-A2-/A24-. In this investigation, we enrolled the 45 patients who were HLA-A2+ or HLA-A24+.

We determined the presence of CTLs in the PBMCs of the CH and LC patients by ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides (Fig. 1, Table I). The representative data of the ELISPOT assay are highlighted. Interestingly, in the CH4 patient, the spots and areas were highly developed in the GPC3 A2-3 and A2-4 peptide-stimulated PBMCs (Fig. 1A). However, few spots and areas were detected in the negative control (no peptide). In addition, GPC3 A24-8 peptide-restricted CTLs were also detected in the CH5 and LC5 patients (Fig. 1B and C). These results suggest that GPC3-specific CTLs are present in the PBMCs of some of CH and LC patients.

Cytotoxicity of CTLs induced by stimulation with the GPC3 (A2-3) peptide. To clarify the cytotoxic activity of GPC3-specific CTLs induced by stimulation with the GPC3 peptide, the HCC cell line, SK-Hep-1/GPC3, transfected with GPC3 and expressing HLA-A2 and HLA-A24 were used as the target cells (Fig. 1D). The CTLs induced from the PBMCs of CH4 (Table I) patient by stimulation with the GPC3 A2-3 peptide showed specific cytotoxicity against the SK-Hep-1/GPC3 and HepG2 cells. On the other hand, no GPC3-specific cytotoxicity was observed against the HLA-classI- K562 cells. These results indicate that GPC3-peptide-specific CTLs induced from CH4 (Table I) patient are cytotoxic against the GPC3-expressing target HCC cells.

Frequency of HLA-A2+ or HLA-A24+ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in PBMC The frequency of patients with GPC3-specific CTLs in their PBMCs is shown in Fig. 2, while the clinical backgrounds of the CH, LC and HCC patients are summarized in Table II. CTL positivity was observed in 5 of 26 CH patients (19%), 5 of 19 LC patients (26%), and 21 of 54 HCC patients (39%). In addition, the percentage of CTL-positive patients tended to

Table I. Detection of GPC3-specific CTLs in the PBMCs of chronic hepatitis/liver cirrhosis patients by ELISPOT assay.

	Peptide/Peptide sequence									
	GPC3 A2-1/RLQPGLKWV		GPC3 A2-3/FVGEFFTDV		GPC3 A2-4/YILGSDINV		GPC3 A24-8/RYILSLEEL		No peptide	
	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)
CH[a]1(A*0201)	1.0±0.0[c]	25905.0±8487.8	2.0±1.0	2826.0±3079.5	1.6±1.1	13895.0±4486.8	NT	NT	0.0±0.0	0.0±0.0
CH2 (A*0201)	1.0±1.7	707.0±1223.6	1.6±1.1	6830.0±6934.2	2.6±1.1	3297.0±3263.1	NT	NT	0.0±0.0	0.0±0.0
CH3 (A*0201)	NT[d]	NT	18.3±5.5	85100.0±17050.1	15.6±2.5	20173.0±4728.4	NT	NT	8.0±1.7	8045.0±1849.1
CH4 (A*0201)	NT	NT	12.0±2.6	55187.0±8618.4	10.0±3.4	22832.0±7632.2	NT	NT	1.0±0.0	3853.0±375.2
CH5 (A*2402)	NT	NT	NT	NT	NT	NT	13.3±3.7	101736.0±54505.9	7.0±1.0	36502.5±14892.4
LC[b]1 (A*0201)	1.0±0.0	1060.0±815.7	2.1±0.2	2944.0±815.7	6.3±0.5	50162.0±4283.0	NT	NT	0.5±0.0	354.0±0.0
LC2 (A*0201)	24.0±3.0	55891.2±23304.1	8.0±2.0	45971.9±25440.5	8.0±1.0	103961.4±13618.6	NT	NT	4.3±0.5	2098.3±2166.5
LC3 (A*0201)	1.3±0.5	2355.0±2855.2	3.6±1.5	8007.0±6564.4	11.3±5.7	100323.0±70946.1	NT	NT	2.0±3.4	2826.0±4894.7
LC4 (A*2402)	NT	NT	NT	NT	NT	NT	14.0±8.0	41331.0±31472.6	3.0±0.0	7065.0±3996.5
LC5 (A*2402)	NT	NT	NT	NT	NT	NT	35.3±2.3	200882.0±21210.9	8.3±2.3	8714.0±2855.5

[a]CH, chronic hepatitis. [b]LC, liver cirrhosis. [c]We show values higher than the value for 'No peptide' by a bold font. [d]NT, not tested.

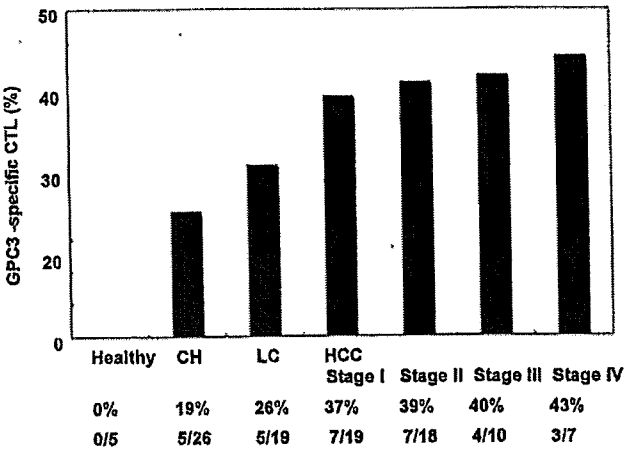

Figure 2. Frequency of HLA-A2[+] or HLA-A24[+] CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in the PBMCs. GPC3-peptide-specific CTLs were detected in 19 and 26% of the patients with CH and LC, respectively. In the HCC patients, the percentage of these CTLs tended to increase with increasing stage of progression of the disease: 37% (stage I), 39% (stage II), 40% (stage III) and 43% (stage IV).

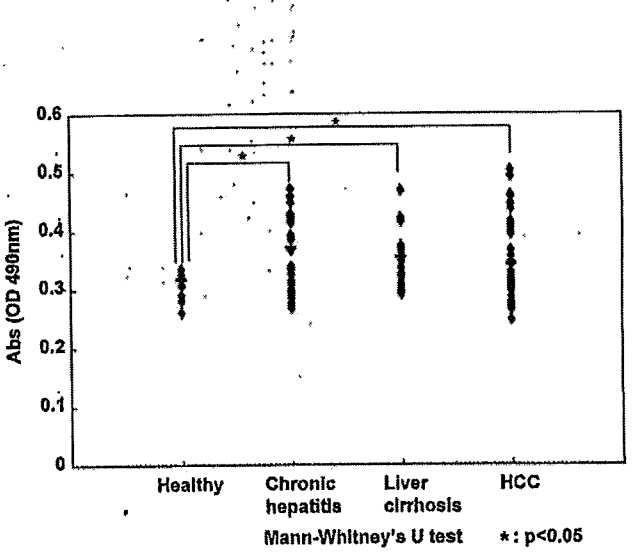

Figure 3. Plasma titers of anti-GPC3 IgG in the CH, LC and HCC patients. Anti-GPC3 IgG was detected by ELISA using recombinant GPC3 protein. A significantly higher titer of IgG to GPC3 was observed in the CH ($p<0.05$), LC ($p<0.05$) and HCC patients ($p<0.05$) as compared with that in healthy donors. *$p<0.05$ (Mann-Whitney U test).

increase with increasing clinical stage of HCC; stage I (7/19, 37%), stage II (7/18, 39%), stage III (4/10, 40%), and stage IV (3/7, 43%) (Table II). There were no CTL-positive cases (0/5, 0%) in healthy donors.

Anti-GPC3 IgG in the plasma in patients with CH, LC and HCC. To examine the quantitative titers of anti-GPC3 IgG in the plasma of patients with CH, LC and HCC, we carried out ELISA using the recombinant GPC3 protein (Fig. 3). The titers in the CH, LC and HCC patients were significantly higher as compared with the peak titer in healthy controls. These results indicate that the GPC3 antigen is expressed not only in HCC patients, but also in CH and LC patients.