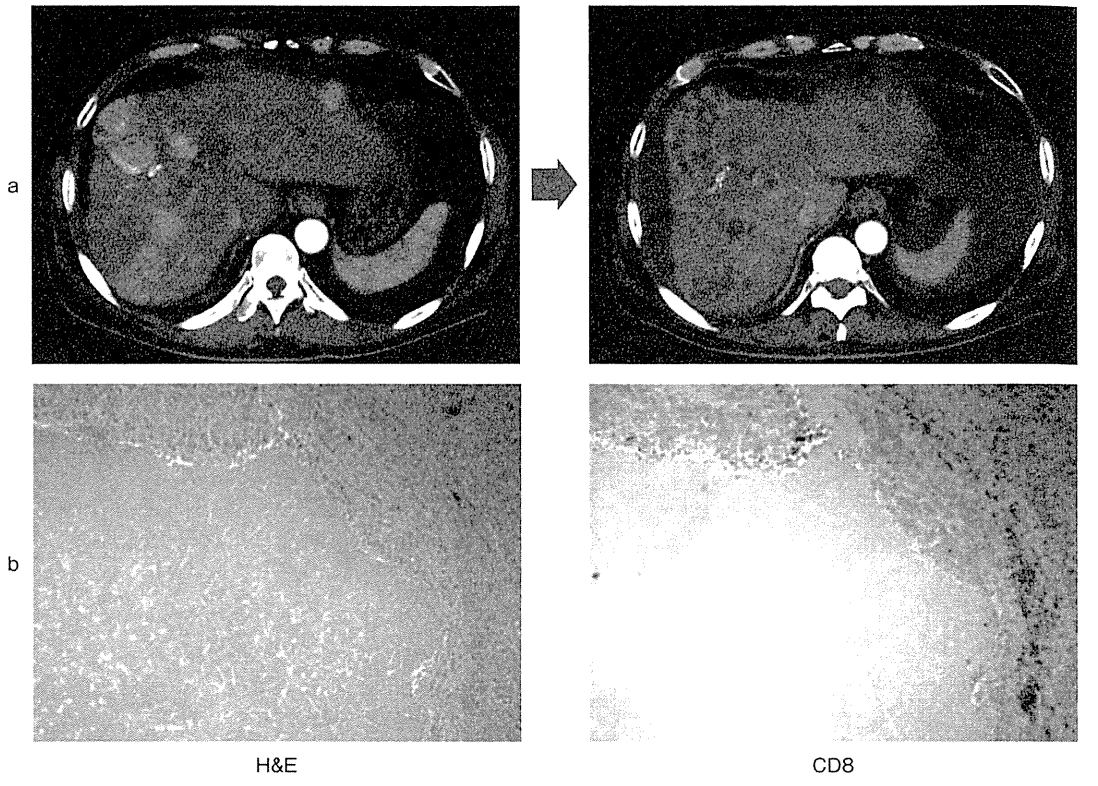

〔文献3)より引用・改変〕

図3　GPC3ペプチドワクチンによる抗腫瘍効果を認めた症例

a：腹部造影CT
　左：GPC3ペプチドワクチン投与前。両葉に多発する肝癌を認め，腫瘍は濃染像を呈している
　右：GPC3ペプチドワクチン2回投与後。両葉の多発腫瘍は大部分で低吸収域となり，壊死像を呈している
b：病理組織所見（剖検）
　左：H＆E染色では，広範な肝細胞癌の壊死像を認め，その辺縁の一部に残存腫瘍細胞を認める
　右：免疫染色では，残存腫瘍細胞部に一致してCD8$^+$T細胞（CTL）の浸潤を認める

なり得る治療法である。肝癌に対するGPC3ペプチドワクチン療法は臨床試験の結果を受け企業への導出がなされ，GPC3ペプチドを含むカクテルワクチンの企業治験がスタートした。さらなる基礎および臨床研究の展開により，第4の癌治療法としての確立が期待される。

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Large-scale expansion of γδ T cells and peptide-specific cytotoxic T cells using zoledronate for adoptive immunotherapy

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Abstract. Specific cellular immunotherapy for cancer requires efficient generation and expansion of cytotoxic T lymphocytes (CTLs) that recognize tumor-associated antigens. However, it is difficult to isolate and expand functionally active T-cells *ex vivo*. In this study, we investigated the efficacy of a new method to induce expansion of antigen-specific CTLs for adoptive immunotherapy. We used tumor-associated antigen glypican-3 (GPC3)-derived peptide and cytomegalovirus (CMV)-derived peptide as antigens. Treatment of human peripheral blood mononuclear cells (PBMCs) with zoledronate is a method that enables large-scale γδ T-cell expansion. To induce expansion of γδ T cells and antigen-specific CTLs, the PBMCs of healthy volunteers or patients vaccinated with GPC3 peptide were cultured with both peptide and zoledronate for 14 days. The expansion of γδ T cells and peptide-specific CTLs from a few PBMCs using zoledronate yields cell numbers sufficient for adoptive transfer. The rate of increase of GPC3-specific CTLs was approximately 24- to 170,000-fold. These $CD8^+$ cells, including CTLs, showed GPC3-specific cytotoxicity against SK-Hep-1/hGPC3 and T2 pulsed with GPC3 peptide, but not against SK-Hep-1/vec and T2 pulsed with human immunodeficiency virus peptide. On the other hand, $CD8^-$ cells, including γδ T cells, showed cytotoxicity against SK-Hep-1/hGPC3 and SK-Hep-1/vec, but did not show GPC3 specificity. Furthermore, adoptive cell transfer of $CD8^+$ cells, $CD8^-$ cells, and total cells after expansion significantly inhibited tumor growth in an NOD/SCID mouse model. This study indicates that simultaneous expansion of γδ T cells and peptide-specific CTLs using zoledronate is useful for adoptive immunotherapy.

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Key words: adoptive cell transfer, cytotoxic T lymphocyte, γδ T cell, glypican-3, hepatocellular carcinoma

Introduction

Current therapeutic options for cancer treatment, including surgery, radiotherapy and chemotherapy, have made advancements in recent years and the survival rate of patients with cancer has gradually improved. However, these therapies remain far from satisfactory in most cancers (1,2). Therefore, the development of novel treatment modalities, including antigen-specific cancer immunotherapies with peptide vaccines, dendritic cell vaccines and adoptive cell transfer therapies, is critical for the further advancement of effective cancer treatments (3-5).

We found that glypican-3 (GPC3), which is an oncofetal antigen that is overexpressed in human hepatocellular carcinoma (HCC), was shown to be a useful target antigen for immunotherapy in several studies (6-10). Based on results obtained from preclinical studies, we conducted a phase I clinical trial using a GPC3-derived peptide vaccine in 33 patients with advanced HCC. In almost all vaccinated patients, the frequency of GPC3 peptide-specific CTLs increased after vaccination. Furthermore, this was the first study to show that the frequency of peptide-specific CTLs was correlated with overall survival in patients with HCC receiving peptide vaccines (11,12). Although the peptide vaccine is a potentially attractive treatment modality, the antitumor effects of the peptide vaccine alone are not dramatic in patients with advanced HCC. Therefore, the establishment of an innovative strategy to enhance the power of antigen-specific cancer immunotherapy is urgently required.

Cellular immunotherapy of solid and hematopoietic malignancies is regarded as a promising approach to treat relapse after or resistance to conventional treatments. The adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) results in objective cancer regression in 49 to 72% of patients with metastatic melanoma (13). However, due to the scarcity of TILs, this therapy is only possible for a limited number of patients. It is difficult to isolate and expand functionally active T cells. Development of a new method of CTL expansion may be useful in addressing this problem.

It was recently reported that γδ T cells are attractive mediators of cancer immunotherapy (14). Several clinical studies that included manipulation of γδ T cells by aminobisphosphonate administration or adoptive transfer of γδ

T cells were performed (15-17). γδ T cells recognize their targets independently of major histocompatibility complex (MHC)-mediated antigen presentation (18-21). Human γδ T cells kill a vast repertoire of tumor cell lines and primary samples *in vitro*, including leukemia, lymphoma, melanoma, neuroblastoma and multiple types of carcinomas (22-25). In addition, human γδ T cells mediate antibody-dependent cellular cytotoxicity (26,27). On the other hand, activated human γδ T cells produce large amounts of interferon-γ (28,29), a central cytokine in antitumor immune responses. Moreover, it has been reported that zoledronate stimulates proliferation of γδ T cells, which then stimulate CTLs as antigen-presenting cells (APCs) (30-33). Therefore, we have attempted to use γδ T cells as both effector cells and APCs.

We report on the development of a more effective adoptive immunotherapy. We investigated a new method to induce expansion of γδ T cells and peptide-specific CTLs using zoledronate.

Materials and methods

Patient samples. Patient blood samples were obtained during the performance of clinical trials at National Cancer Center Hospital East. We carried out two clinical trials involving GPC3-derived peptide vaccine. The phase I trial was carried out among 33 patients with advanced or metastatic HCC from February, 2007 to November, 2009 (11,12). The trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR no. 000001395). We subsequently conducted a phase II trial involving the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC. Forty patients with initial HCC who had undergone surgery or radiofrequency ablation were enrolled in this phase II trial (UMIN-CTR no. 000002614). These patients were enrolled after providing a written informed consent. Patients were intradermally injected with HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) or HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide vaccine emulsified with incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC, Paris, France). This study was approved by the Ethics Committee of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

PBMCs. Peripheral blood (30 ml) was obtained from healthy volunteers or patients at the times designated in the protocol (before the first vaccination and 2 weeks after each vaccination). Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll density gradient centrifugation from buffy coats. In this study, we used the remaining PBMCs after immunological monitoring in the clinical trials.

Cell lines. The human liver cancer cell lines SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02) and SK-Hep-1/hGPC3 ($GPC3^+$, HLA-A*02:01/A*24:02) were used as target cells. SK-Hep-1/hGPC3 is an established stable GPC3-expressing cell line transfected with a human GPC3 gene, and SK-Hep-1/vec is an established counterpart cell line in which an empty vector was transfected. T2 (HLA-A*02:01, TAP^-) and T2A24 (HLA-A*02:01/A*24:02, TAP^-) cells were pulsed with GPC3 peptide or human immunodeficiency (HIV) peptide at room temperature for 1 h. They were conserved in our laboratory. Cells were cultured at 37°C in RPMI-1640 or DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2.

Synthetic peptides. The peptides used in this study were as follows: HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide (American Peptide Company, Sunnyvale, CA), HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) peptide (American Peptide Company), HLA-A*02:01-restricted cytomegalovirus $(CMV)_{495-503}$ (NLVPMVATV) peptide (ProImmune, Rhinebeck, NY, USA), HLA-A*24:02-restricted $CMV_{341-349}$ (QYDPVAALF) peptide (ProImmune), and HLA-A*02:01-restricted HIV_{77-85} (SLYNTYATL) peptide (ProImmune). The peptides were dissolved and diluted in 7% $NaHCO_3$ or dimethyl sulfoxide.

Large-scale expansion using zoledronate. PBMCs were cultured ($2x10^6$ cells/well) with zoledronate (5 μM) (Novartis Pharma, Basel, Switzerland) and CMV or GPC3 peptide (10 μM) in AIM-V medium (Gibco) supplemented with 10% human AB serum (Sigma) and recombinant human interleukin (IL)-2 (1,000 IU/ml) (Novartis Pharma) for 14 days. The stimulation procedure was performed at 37°C and 5% CO_2. Scale-up of cells was performed in accordance with their growth.

Expansion of peptide-specific CTLs in the absence of zoledronate. To obtain zoledronate-activated γδ T cells, PBMCs were stimulated with zoledronate and IL-2 for 7 days. On day 7, zoledronate-activated γδ T cells were sorted using FACSAria II. $CD8^+$ cells and γδ T cells without zoledronate activation were sorted from non-cultured PBMCs using microbeads and FACSAria II, respectively. Dendritic cells (DCs) were induced from $CD14^+$ cells using GM-CSF and IL-4. On day 5, DCs were stimulated with TNF-α for 2 days. We used γδ T cells with or without zoledronate activation and TNF-α-stimulated DCs as stimulator cells. Stimulator cells were pulsed with CMV peptide (10 μM) for 1 h at room temperature. After washing out the peptide, stimulator cells were co-cultured for 2 weeks with responder $CD8^+$ cells and the addition of IL-2 in the absence of zoledronate. We compared the percentages of CMV peptide-specific CTLs in responder $CD8^+$ cells using dextramer assays.

In vitro stimulation of GPC3 peptide-specific CTL clones. GPC3 peptide-specific CTL clones were previously generated by single cell sorting using a GPC3-dextramer or CD107a antibody. CTL clones were stimulated as described previously (34).

Dextramer staining and flow cytometry analysis. The PBMCs were stained with CMV, GPC3 or HIV Dextramer-RPE (Immudex, Copenhagen, Denmark) for 10 min at room temperature and with anti-CD8-FITC (ProImmune) or anti-CD8-APC (BioLegend, San Diego, CA), anti-CD45RA-FITC (BD Biosciences, San Jose, CA, USA), and anti-CCR7-PerCP/Cy5.5 (BioLegend) for 20 min at 4°C. To detect γδ T cells, PBMCs were stained with anti-TCR-Vγ9-FITC (Beckman Coulter, Erembodegem,

Table I. Rate of increase in the number of cells in 16 patients with HCC.

		Total			γδ			GPC3 specific CTLs		
Sample	HLA-A	Day 0	Day 14	The rate of increase	Day 0	Day 14	The rate of increase	Day 0[a]	Day 14[b]	The rate of increase
1	02:01	$2.0x10^6$	$1.7x10^8$	85	$1.2x10^4$	$2.8x10^7$	$2.3x10^3$	$2.0x10^3$	$6.1x10^7$	$3.1x10^4$
2	02:01	$2.0x10^6$	$5.4x10^8$	$2.7x10^2$	$2.8x10^4$	$3.1x10^8$	$1.1x10^4$	$1.6x10^2$	$2.7x10^7$	$1.7x10^5$
3	02:01	$2.0x10^6$	$1.7x10^8$	85	$8.8x10^3$	$7.8x10^7$	$8.9x10^3$	92	$1.0x10^6$	$1.1x10^4$
4	02:01	$2.0x10^6$	$1.7x10^8$	85	$2.4x10^4$	$9.5x10^7$	$4.0x10^3$	$1.3x10^3$	$1.9x10^7$	$1.5x10^4$
5	02:01	$2.0x10^6$	$7.4x10^7$	37	$9.4x10^3$	$5.2x10^7$	$5.5x10^3$	92	$1.8x10^5$	$2.0x10^3$
6	02:01	$2.0x10^6$	$2.5x10^7$	13	$2.0x10^4$	$1.2x10^7$	$6.0x10^2$	$1.0x10^3$	$1.9x10^6$	$1.9x10^3$
7	02:01	$2.0x10^6$	$1.6x10^8$	80	$1.1x10^4$	$7.4x10^7$	$6.7x10^3$	$2.1x10^2$	$4.4x10^6$	$2.1x10^4$
8	02:01	$2.0x10^6$	$4.0x10^8$	$2.0x10^2$	$1.2x10^4$	$1.3x10^8$	$1.1x10^4$	$4.0x10^2$	$2.6x10^7$	$6.5x10^4$
9	02:01	$2.0x10^6$	$4.0x10^7$	20	$8.0x10^3$	$4.4x10^6$	$5.5x10^2$	$8.4x10^2$	$5.9x10^6$	$7.0x10^3$
10	02:01	$2.0x10^6$	$2.2x10^6$	1.1	$2.0x10^3$	$4.5x10^4$	23	92	$2.2x10^3$	24
11	02:01	$2.0x10^6$	$1.5x10^8$	75	$6.0x10^3$	$4.8x10^7$	$8.0x10^3$	$3.0x10^2$	$2.0x10^7$	$6.7x10^4$
12	02:01	$2.0x10^6$	$1.1x10^8$	55	$6.0x10^3$	$1.2x10^7$	$2.0x10^3$	$1.2x10^3$	$3.5x10^7$	$2.9x10^4$
13	24:02	$2.0x10^6$	$5.8x10^6$	2.9	$1.8x10^4$	$3.8x10^6$	$2.1x10^2$	$1.3x10^2$	$6.3x10^4$	$4.9x10^2$
14	24:02	$2.0x10^6$	$4.0x10^6$	2	$2.2x10^4$	$2.6x10^6$	$1.2x10^2$	$1.0x10^2$	$3.1x10^4$	$3.1x10^2$
15	24:02	$2.0x10^6$	$9.9x10^7$	50	$3.8x10^3$	$3.8x10^7$	$1.0x10^4$	$1.8x10^2$	$1.2x10^6$	$6.7x10^3$
16	24:02	$2.0x10^6$	$4.0x10^7$	20	$9.8x10^3$	$5.6x10^6$	$5.7x10^2$	$1.4x10^2$	$1.4x10^5$	$1.0x10^3$

[a]Frequency of GPC3-specific CTLs of $2x10^6$ PBMCs was measured by *ex vivo* IFN-γ ELISPOT assay. [b]GPC3-specific CTLs after cell culture were measured by flow cytometry.

Belgium) and anti-CD3-PC5 (BioLegend) for 20 min at 4°C. γδ T cells, with or without zoledronate activation, and TNF-DCs were stained with anti-HLA-class I-FITC, anti-CD80-FITC, anti-CD83-FITC and anti-CD86-PE (BD Biosciences) antibodies for 20 min at 4°C. Flow cytometry analysis was carried out using FACSCanto II (BD Biosciences).

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech, Tokyo, Japan) as described previously (34). Target cells were labeled with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37°C. The labeled cells were then incubated with effector cells for 4 to 6 h. As effector cells, $CD8^+$ and $CD8^-$ T cells were isolated using human CD8 microbeads (BD Bioscience) from PBMCs stimulated for 14 days. Assays were conducted in duplicate.

Transfer of effector cells to NOD/SCID mice implanted with the GPC3$^+$ or GPC3$^-$ cell line. Female NOD/SCID (6-8 weeks old) were purchased from Japan Charles River Laboratories (Yokohama, Japan). All animal procedures were performed according to the guidelines for the Animal Research Committee of the National Cancer Center, Japan. We inoculated SK-Hep-1/hGPC3 or SK-Hep-1/vec cells subcutaneously into the right flank of NOD/SCID mice. We intravenously injected the $CD8^+$ cells, $CD8^-$ cells, or both, as effector cells. We injected PBS as a negative control. Before adoptive transfer, we examined the percentage of $CD8^+$ cells in expanded cells using flow cytometry. The percentage of $CD8^+$ cells after expansion was ~25% of all cells. We injected immune cells at this ratio. We injected $5x10^6$ cells per mouse for the $CD8^+$-cell-treatment group. We injected $1.5x10^7$ cells per mouse for the $CD8^-$ cell treatment group and $2.0x10^7$ cells per mouse for the all cells treatment group. We performed adoptive cell transfer of expanded cells using five mice per group. The tumor volume was monitored and calculated using the following formula: tumor volume (mm^3) = a x b^2 x 0.5, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid.

Statistical analysis. The correlation between the number of GPC3-specific CTLs and γδ T cells at days 0 and 14 was analyzed using the Spearman's rank correlation coefficient. Comparisons of tumor volume at the last time point were performed using the Mann-Whitney U test. Differences were considered significant at $P<0.05$.

Results

Zoledronate induces expansion of γδ T cells and peptide-specific CTLs from PBMCs. To assess whether this new culture method can induce the expansion of γδ T cells and peptide-specific CTLs, PBMCs were stimulated once with zoledronate and an antigen-derived peptide. Fig. 1A shows the representative data using PBMCs from a healthy volunteer. The number of total cells increased $3.2x10^2$-fold after 14 days (from $2.0x10^6$ to $6.4x10^8$). In flow cytometry analysis, γδ T cells increased $8.0x10^3$-fold after 14 days [from $5.6x10^4$ (2.8%) to $4.5x10^8$ (70%)]. Simultaneously with γδ T cells, CMV

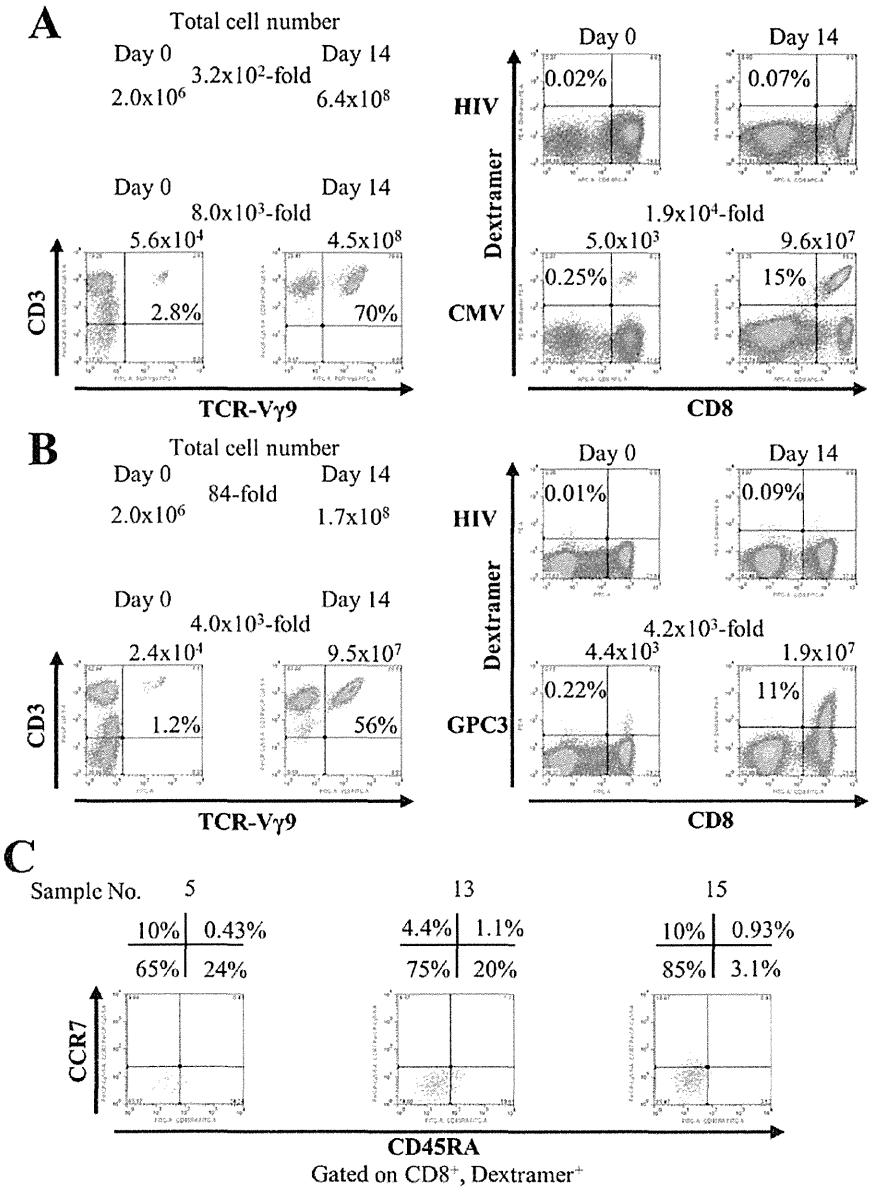

Figure 1. Zoledronate induces expansion of γδ T cells and peptide-specific CTLs. We performed flow cytometry analysis before (day 0) and after (day 14) cell culture. Representative data are shown. (A) PBMCs from a healthy volunteer were stimulated with CMV-derived peptide and zoledronate. (B) PBMCs from a patient vaccinated with GPC3 peptide were stimulated with GPC3-derived peptide and zoledronate. The number indicates the number of cells. The presence of TCR-Vγ9$^+$, CD3$^+$ cells indicated γδ T cells. The presence of CD8$^+$, dextramer$^+$ cells indicated antigen-specific CTLs. HIV-dextramer was used as a negative control. (C) Analysis of the phenotype of CD8$^+$, GPC3-dextramer$^+$ cells at day 14. The CD45RA$^-$, CCR7$^-$ phenotype indicated the effector memory phenotype.

peptide-specific CTLs increased 1.9x10^4-fold after 14 days [from 5.0x10^3 (0.25%) to 9.6x10^7 (15%)]. Similar results were obtained from three healthy subjects (data not shown).

Next, we investigated the capacity of this culture method to induce expansion of CTLs specific for peptides derived from the weakly immunogenic tumor-associated self-antigen GPC3. PBMCs from vaccinated patients were stimulated once with zoledronate and a GPC3-derived peptide. In Fig. 1B, the number of total cells increased 84-fold after 14 days (from 2.0x10^6 to 1.7x10^8). γδ T cells increased 4.0x10^3-fold after 14 days [from 2.4x10^4 (1.2%) to 9.5x10^7 (56%)]. GPC3 peptide-specific CTLs increased 4.2x10^3-fold after 14 days [from 4.4x10^3 (0.22%) to 1.9x10^7 (11%)]. In addition, during expansion, GPC3 peptide-specific CTLs acquired mainly an effector memory phenotype (CD45RA$^-$, CCR7$^-$) (Fig. 1C). One of the features of this culture method is the rate of increase in the number of cells. Table I shows the rate of increase in the number of cells in 16 patients with HCC. We found that the total cell number increased (range, 1.1-270-fold), γδ T cells increased (range, 23-1.1x10^4-fold), and GPC3 peptide-specific CTLs increased (range, 24-1.7x10^5-fold) after 14 days. These results suggest that peptide-specific CTLs were successfully expanded with the proliferation of γδ T cells from PBMCs by this new culture method.

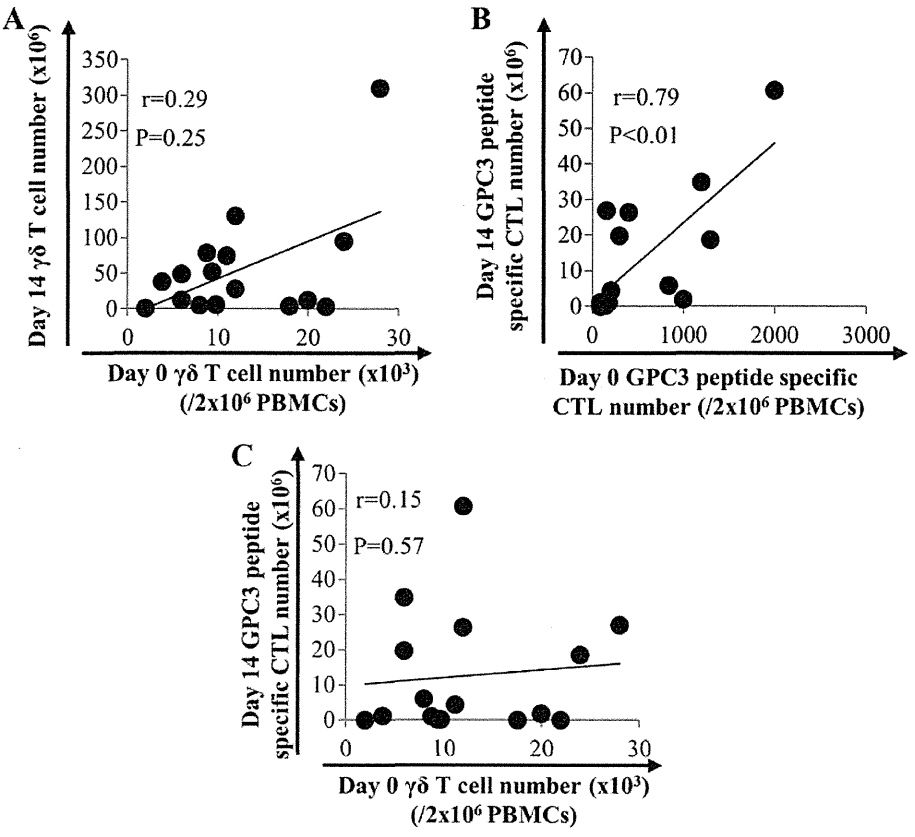

Figure 2. Efficacy of the method to induce expansion of GPC3 peptide-specific CTLs. (A) The correlation between the number of γδ T cells before and after expansion (n=16). (B) The correlation between the number of GPC3 peptide-specific CTLs before and after expansion. The number of GPC3 peptide-specific CTLs after expansion was correlated with that before expansion (n=16). (C) The correlation between the number of γδ T cells and the number of GPC3 peptide-specific CTLs after expansion (n=16).

Efficiency of the culture method to induce expansion of GPC3 peptide-specific CTLs. One of the problems of cell transfer therapy is that it cannot predict cell growth prior to cell culture. Therefore, to identify predicting factors, we investigated the ability of this culture method to induce expansion of γδ T cells and GPC3 peptide-specific CTLs in 16 patients with HCC. As shown in Fig. 2, the number of γδ T cells after expansion did not correlate with that before expansion (Fig. 2A). On the other hand, the number of GPC3 peptide-specific CTLs after expansion correlated with that before expansion ($P<0.01$, $r=0.79$) (Fig. 2B). This result indicates that the number of GPC3 peptide-specific CTLs before expansion is a predicting factor. We expected a positive correlation between the number of γδ T cells and the number of GPC3 peptide-specific CTLs after expansion. However, no such correlation was observed (Fig. 2C).

Activated γδ T cells function as antigen-presenting cells. To examine whether the expansion of peptide-specific CTLs is enhanced by simultaneous activation/expansion of γδ T cells, we expanded peptide-specific CTLs in the absence of zoledronate. The purity of sorted $CD8^+$ cells and γδ T cells with or without zoledronate activation was greater than 99% (Fig. 3A). The expansion of peptide-specific CTLs stimulated by γδ T cells with zoledronate activation (70.8%) was higher than by γδ T cells without zoledronate activation (43.6%). Moreover, the CTL-expanding ability of zoledronate-activated γδ T cells was comparable to that of TNF-DCs (62.0%), which are known professional antigen-presenting cells. These results indicate that zoledronate-activated γδ T cells function as antigen-presenting cells in co-cultures in the absence of zoledronate (Fig. 3B). We compared cell surface expression of antigen-presenting molecules and co-stimulatory molecules on γδ T cells (with or without zoledronate activation) and TNF-DCs. All cells expressed HLA-class I; however, γδ T cells without zoledronate activation did not express co-stimulatory molecules. Furthermore, CD86 expression in zoledronate-activated γδ T cells was comparable with that of TNF-DCs (Fig. 3C). These results indicate that γδ T cells activated by zoledronate acquire antigen-presenting properties accompanied by CD86 expression.

Cytotoxic activity of expanded cells. We performed a cytotoxicity assay to assess the peptide specificity and cytotoxic activity of expanded cells against cancer cells. We used $CD8^+$ and $CD8^-$ cells that were isolated from cultured cells using CD8 microbeads at day 14 as effector cells. The purity of $CD8^+$ cells was 99.4%. We performed further immunophenotyping of $CD8^-$ cells. $CD3^+$ $Vg9^+$ cells were 80.0% of $CD8^-$ cells. $CD8^-$ cells also included $CD3^+$ $CD4^+$ cells (4.1%), $CD3^+$ $CD8^+$ cells (9.4%), and $CD3^-$ $CD56^+$ cells (NK cells; 3.6%). $CD14^+$ cells (monocytes; 0.1%) and

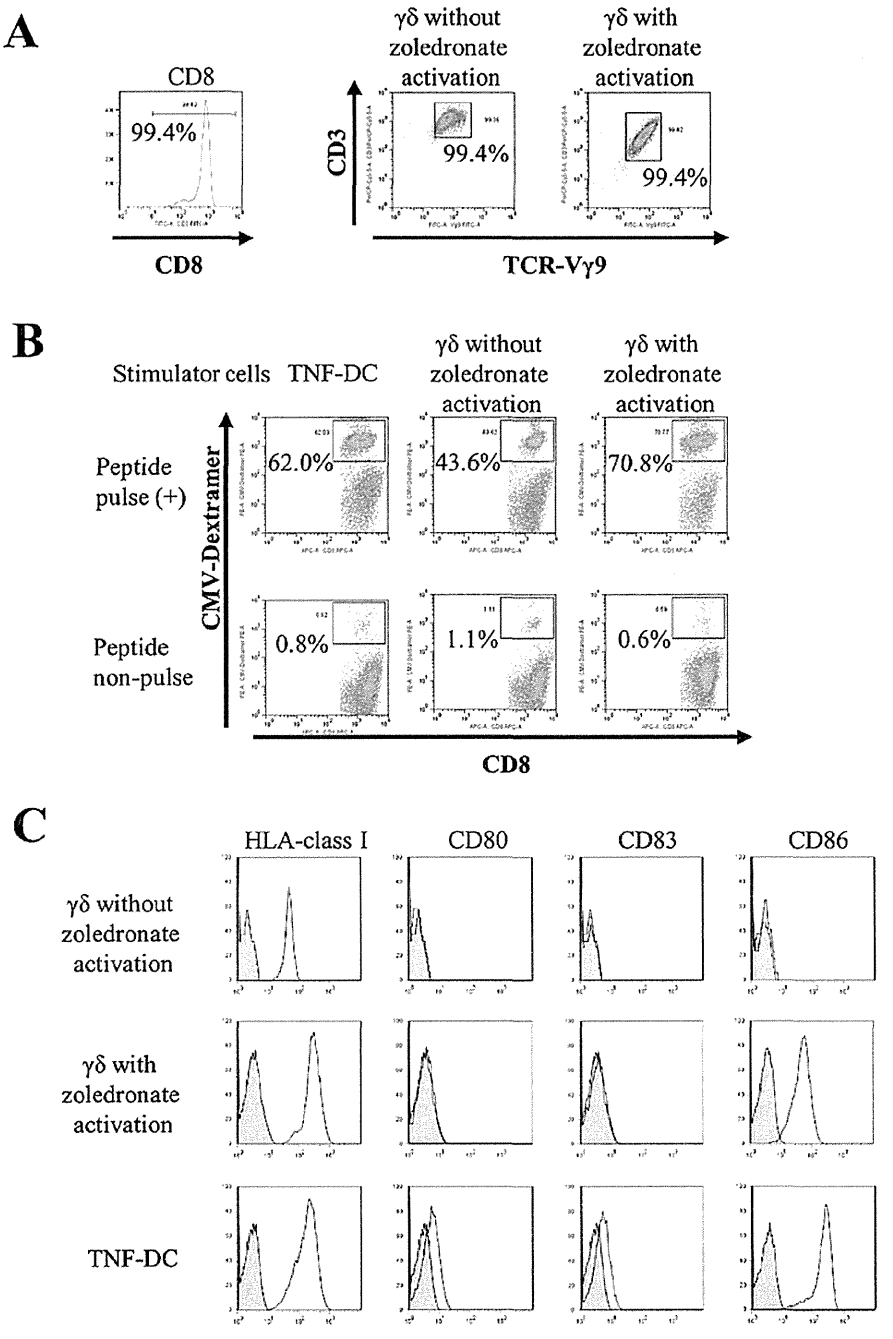

Figure 3. Activated γδ T cells function as antigen-presenting cells. (A) The percentages of sorted cells were analyzed using flow cytometry. The purity of sorted $CD8^+$ cells, γδ T cells without zoledronate activation and γδ T cells with zoledronate activation were greater than 99%. (B) The responder $CD8^+$ cells were co-cultured with stimulator cells pulsed with CMV peptide in the absence of zoledronate. After 2 weeks, flow cytometry analyses were performed using CMV-Dextramer. Non-pulsed stimulator cells were co-cultured with responder $CD8^+$ as negative controls. Representative data are shown. Similar results were obtained from three healthy subjects. (C) Cell surface expression of antigen-presenting molecules (HLA-class I) and co-stimulatory molecules (CD80, CD83 and CD86) on γδ T cells (with or without zoledronate activation) and TNF-DCs using flow cytometry. Black line shows a specific antibody. Gray-filled area shows negative control. Representative data are shown. Similar results were obtained from three healthy subjects.

$CD19^+$ cells (B cells; 0.1%) were not observed in $CD8^-$ cells. These results indicate that $CD8^-$ cells were predominantly γδ T cells (Fig. 4A). Similar results were obtained from four patients. $CD8^+$ cells showed cytotoxicity against T2 cells pulsed with GPC3 peptide, whereas $CD8^-$ cells did not show cytotoxicity against T2 cells pulsed with both GPC3 and HIV peptide (Fig. 4B). Moreover, we used SK-Hep-1/hGPC3 cells as target cells; they were transfected with the GPC3 gene and endogenously presented GPC3 peptide. $CD8^+$ cells showed GPC3-specific cytotoxicity, whereas $CD8^-$ cells showed cytotoxicity against SK-Hep-1 cells but did not show GPC3 specificity (Fig. 4C). We performed cytotox-

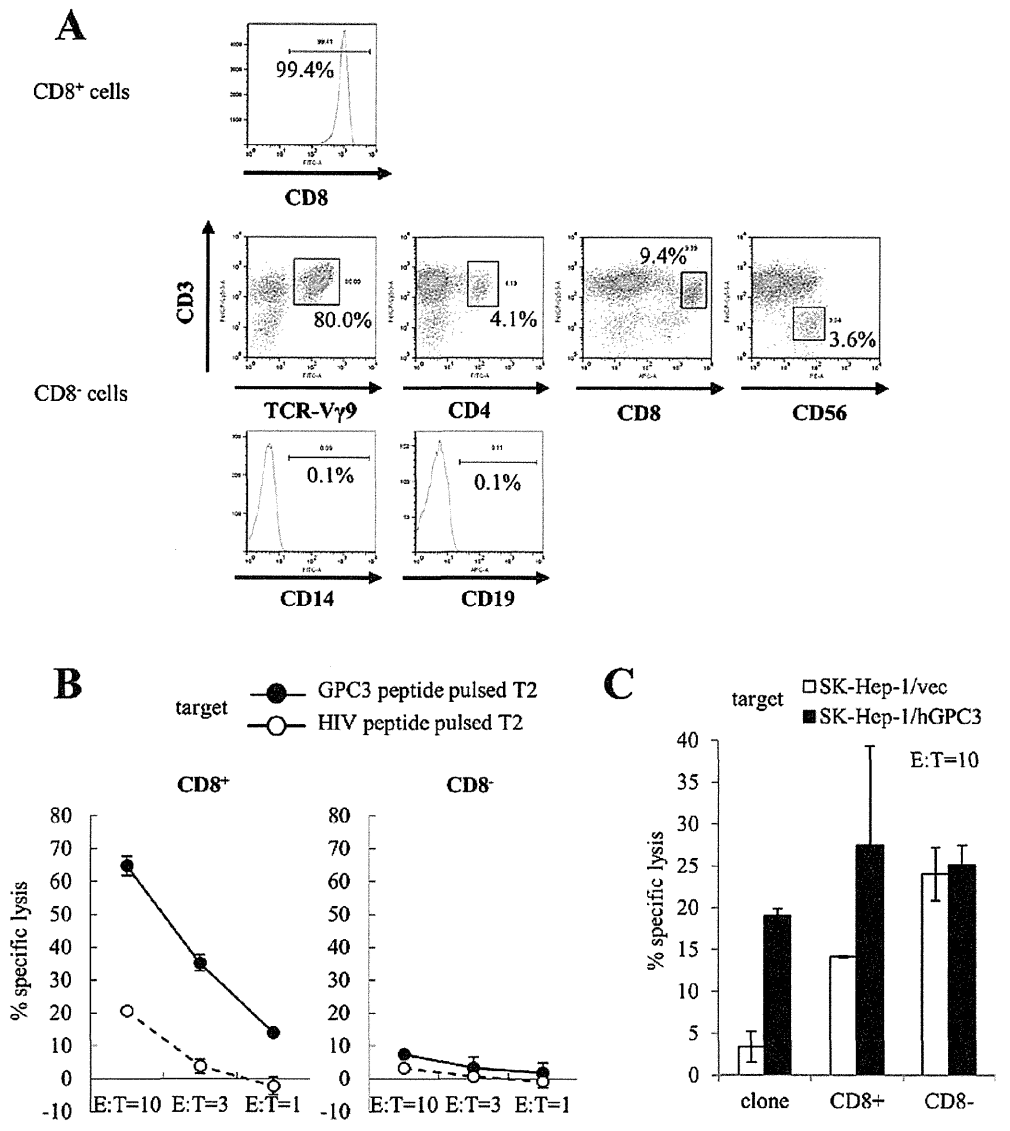

Figure 4. Cytotoxicity assay of cultured cells. We used CD8+ and CD8- cells that were isolated from cultured cells using CD8 microbeads at day 14 as effector cells. A GPC3 peptide-specific CTL clone was used as a positive control. We performed cytotoxicity assays using expanded cells from four patients. Similar results were obtained in three of the four patients. Representative data are shown. (A) We examined the purity of the CD8+ cell populations obtained for these experiments. We performed further immunophenotyping of CD8- cells using flow cytometry. (B) T2 cells pulsed with GPC3 (black circle) or HIV (white circle) peptide were used as target cells. CD8+ cells (left) showed GPC3 peptide-specific cytotoxic activity. CD8- cells (right) did not show GPC3 peptide-specific cytotoxic activity. (C) SK-Hep-1/hGPC3 (black bar) or SK-Hep-1/vec (white bar) cells were used as target cells. CD8+ cells showed GPC3-specific cytotoxicity, whereas CD8- cells showed cytotoxicity against SK-Hep-1 cells, but not GPC3 specificity (E:T=10). Data represent the means ± SD.

icity assays using expanded cells from four patients. Similar results were obtained in three of the four patients. These results indicate that CD8+ cells included mostly GPC3 peptide-specific CTLs that had cytotoxic activity against cancer cells and endogenously presented GPC3 peptide, and CD8- cells included mostly γδ T cells that had cytotoxic activity against cancer cells.

Antitumor activity of γδ T cells and GPC3-specific CTLs in vivo. We performed adoptive cell transfer of expanded cells in a mouse model. We subcutaneously inoculated SK-Hep-1/vec (Fig. 5A) or SK-Hep-1/hGPC3 (Fig. 5B) cell lines into NOD/SCID mice and intravenously injected effector cells twice. As effector cells, we used CD8+ or CD8- cells that were isolated from cultured cells using CD8 microbeads at day 14, and we used all cells that included both CD8+ and CD8- cells. As shown Fig. 5B, the growth of SK-Hep-1/hGPC3 treated with CD8+ or CD8- cells was significantly inhibited compared with the negative control. In addition, treatment of all cells, including both CD8+ and CD8- cells, tended to show an additive inhibitory effect. On the other hand, the growth of SK-Hep-1/vec that was inhibited in the treatment of CD8- or all cells was not inhibited by treatment of CD8+ cells (Fig. 5A). These results indicate that cultured cells had antitumor effects due to the respective CD8+ and CD8- cells.

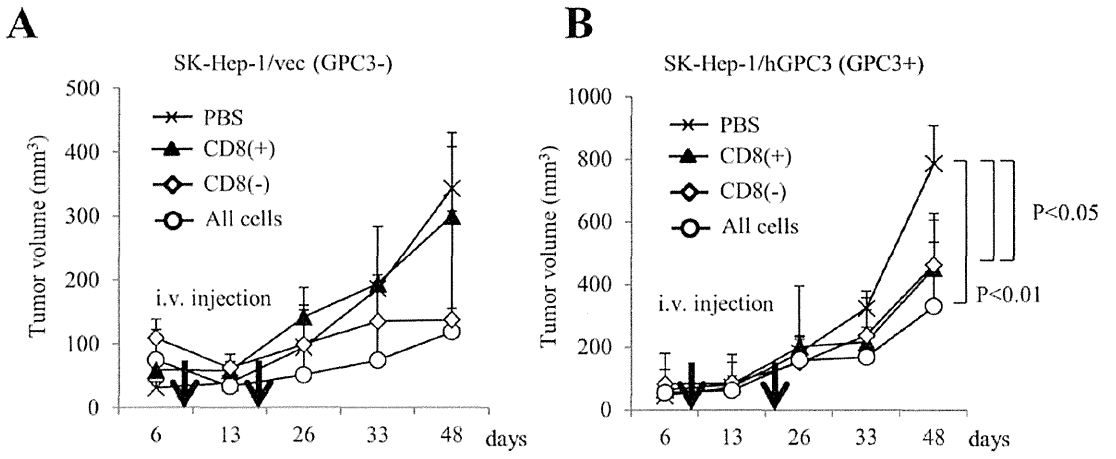

Figure 5. Antitumor activity of γδ T cells and GPC3-specific CTLs *in vivo*. We subcutaneously inoculated (A) SK-Hep-1/vec or (B) SK-Hep-1/hGPC3 cells into NOD/SCID mice and intravenously injected effector cells. We performed adoptive cell transfer of expanded cells using five mice per group. Data represent the means ± SD. (A) The growth of SK-Hep-1/vec treated with $CD8^-$ cells or all cells was significantly inhibited. The growth of SK-Hep-1/vec treated with $CD8^+$ cells was not inhibited. (B) The growth of SK-Hep-1/hGPC3 treated with $CD8^+$ or $CD8^-$ cells was significantly inhibited. In addition, treatment with all cells, including both $CD8^+$ and $CD8^-$ cells, showed an additive inhibitory effect.

Discussion

Specific cellular immunotherapy of cancer requires efficient generation and expansion of CTLs that recognize tumor-associated antigens. ACT with TILs isolated from metastatic melanoma lesions lead to objective tumor regression. However, TILs can be exploited only in melanoma patients with resectable tumors and from which T cells can be expanded *ex vivo*. An alternative approach has been explored for patients with other types of tumor using autologous lymphocytes isolated from peripheral blood. Various clinical trials involving adoptively transferred autologous T cells transduced with a TCR or chimeric antigen receptors have been conducted (35-37). Clinical trials using our culture method should be performed in the future.

The standard approach to generating tumor-specific CTLs is based on antigen presentation by dendritic cells (DCs). Although DCs are the most efficient APCs known so far, serious drawbacks to their use in adoptive immunotherapy exist, including their scarcity in the peripheral blood, their limited expansion and their functional heterogeneity. These limitations have motivated an intense search for alternative sources of APCs. An antigen-presenting function of γδ T cells was suggested by recent observations that upon activation, these cells acquire phenotypic and functional characteristics of professional APCs concomitant with the capacity to induce primary $CD4^+$ and $CD8^+$ T-cell responses to antigens (30-33). To the best of our knowledge, this is the first report of the simultaneous expansion of γδ T cells and antigen-specific CTLs from the PBMCs of patients.

Most adoptive CTL transfer studies in patients with tumors used approximately 10^8 to 10^{11} T cells/m^2 body surface area of the patient (38). The expansion of CTLs from PBMCs of vaccinated patients with advanced HCC yields cell numbers sufficient for adoptive transfer. Theoretically, the number of GPC3-specific CTLs obtained for apheresis (10 L) is, at most, $1.5x10^{11}$ cells.

One reason for the scarcity of adoptive immunotherapy is the individual variability in cell growth. *In vitro*, it is difficult to adequately expand antigen-specific CTLs in most patients with cancer. In addition, cell growth cannot be predicted before culture. Therefore, to identify predicting factors, we investigated the efficacy of this culture method in inducing expansion of GPC3 peptide-specific CTLs in 16 patients with HCC. The prediction of cell growth may enable the implementation of personalized medicine.

In this study, we assessed the expansion of GPC3 peptide-specific CTLs using PBMCs from vaccinated patients with HCC. GPC3 is also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian CCC and lung squamous cell carcinoma (39-43). Adoptive transfer of GPC3 peptide-specific CTLs may also be available for other GPC3-expressing cancers.

This culture method has a limitation. We performed this culture using PBMCs of the same person both before and after vaccination. GPC3 peptide-specific CTLs could be induced from the PBMCs of all patients after vaccinations. However, this method failed to induce GPC3 peptide-specific CTLs from the PBMCs of patients before vaccination. Similarly, GPC3 peptide-specific CTLs could not be induced from the PBMCs of healthy donors (data not shown). These results may have been caused by the low frequency of cancer antigen-specific CTLs in peripheral blood before vaccination. These results suggest that to increase GPC3 peptide-specific CTLs, vaccination is effective before cell culture. On the other hand, with regard to CMV-derived peptide, CMV peptide-specific CTLs could be induced with the proliferation of γδ T cells from the PBMCs of healthy donors by this culture method. This culture method may also be available for other antigens.

Adoptive cell transfer of all cells after expansion, including both CTLs and γδ T cells, significantly inhibited tumor growth in a mouse model. Tumor cells acquire various immune escape mechanisms including loss of antigens or the

HLA-class I molecule. It may be effective to use both CTLs and γδ T cells because they have different antigen recognition abilities. However, we did not confirm that the results were due to either synergy or an additive effect. Because activated γδ T cells produce large amounts of interferon-γ, it may be a synergy effect. Analysis of the mechanisms of the effectiveness of CTLs and γδ T cells is a future challenge.

On the other hand, we previously reported that intratumor peptide injection was an effective method of enhancing tumor cell antigenicity and that it showed an induced antigen-spreading effect *in vivo* (44,45). Moreover, we are investigating the antitumor activity of γδ T cells against HCC cells pretreated with zoledronate. The combination of these pretreatments that enhance tumor cell antigenicity and adoptive immunotherapy using CTLs and γδ T cells may be a useful application for cancer therapy.

In conclusion, this study indicates that simultaneous expansion of γδ T cells and peptide-specific CTLs using zoledronate are useful for adoptive immunotherapy.

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REVIEW

Critical analysis of the potential of targeting GPC3 in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide. The treatment options for patients with advanced HCC are limited, and novel treatment strategies are required urgently. Glypican-3 (GPC3), a member of the glypican family of heparan sulfate proteoglycans, is overexpressed in 72%–81% of HCC cases, and is correlated with a poor prognosis. GPC3 regulates both stimulatory and inhibitory signals, and plays a key role in regulating cancer cell growth. GPC3 is released into the serum, and so might be a useful diagnostic marker for HCC. GPC3 is also used as an immunotherapeutic target in HCC. A Phase I study of a humanized anti-GPC3 monoclonal antibody, GC33, revealed a good safety profile and potential antitumor activity, and a Phase II trial is currently ongoing. In addition, the authors' investigator-initiated Phase I study of a GPC3-derived peptide vaccine showed good safety and tolerability, and demonstrated that the GPC3 peptide-specific cytotoxic T-lymphocyte frequency in peripheral blood correlated with overall survival in HCC patients. A sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway. GPC3 is currently recognized as a promising therapeutic target and diagnostic marker for HCC. This review introduces the recent progress in GPC3 research, from biology to clinical impact.

Keywords: GPC3, hepatocellular carcinoma, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide.[1] HCC patients are often diagnosed at an advanced stage, and so the prognosis is often poor. Currently, surgery or locally ablative treatments such as percutaneous ethanol injection or radiofrequency ablation are the standard treatments for early-stage HCC. However, these treatments are no longer available and options are limited for most patients with advanced HCC.[2] Generally, transarterial chemoembolization or systemic chemotherapy is used. However, these therapeutic approaches are not curative in most patients. Sorafenib, a multi-targeted tyrosine kinase inhibitor, is the only drug that has significantly prolonged the survival of patients with advanced HCC;[3,4] therefore, it has become the standard agent for first-line systemic treatment. However, the incidence of adverse effects is high, and there are no effective second-line treatments for patients who do not respond to sorafenib. Therefore, new treatment strategies for patients with advanced HCC should be established.

To date, several immunotherapeutic clinical trials in patients with advanced HCC have been performed. These studies have shown feasibility and safety, but no dramatic clinical responses.[5,6] Nevertheless, some randomized controlled trials have shown the

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potential to reduce the risk of cancer recurrence in adjuvant settings.[6] Therefore, an immunotherapeutic approach is potentially an attractive treatment option for HCC.

Various tumor antigens for HCC have been identified and investigated as immunotherapeutic targets.[7] GPC3 is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via glycosylphosphatidylinositol (GPI) anchors.[8] Mutations in *GPC3* cause Simpson–Golabi–Behmel syndrome,[9] which is an X-linked disorder characterized by pre- and postnatal overgrowth with visceral and skeletal anomalies. *GPC3*-deficient mice exhibited similar characteristics as Simpson–Golabi–Behmel syndrome patients.[10] GPC3 is overexpressed in 72%–81% of patients with HCC.[11–15] Therefore, GPC3 has been recognized as a potential immunotherapeutic target or diagnostic marker for HCC. This paper reviews the biology of GPC3 and discusses recent advances in GPC3-targeted HCC immunotherapy.

Tumor-associated antigens (TAAs) in HCC

TAA-specific immunotherapy is an attractive strategy because it is associated with fewer adverse events. Therefore, identifying appropriate TAAs is important for the development of TAA-specific cancer immunotherapies. Boon et al initially reported that MAGE-A was a human TAA in a melanoma patient, and that the human immune system could recognize TAA expressing-cancer cells as foreign bodies and exclude them.[16] Subsequently, a novel approach termed serological analysis of recombinant complementary DNA expression libraries (SEREX) was developed to identify TAAs.[17,18] Complementary DNA microarray technology is also useful for identifying novel cancer-associated genes and for classifying human cancers at the molecular level.[19,20] In HCC, some TAAs, such as AFP, MAGE-A, NY-ESO-1, SSX2, and telomerase reverse transcriptase, have been identified.[7] Although GPC3 is overexpressed in HCC,[11–15] it is not expressed in most normal adult tissues. Furthermore, GPC3-expression was correlated with poor prognosis in patients with HCC: GPC3-positive HCC patients had a significantly lower 5-year survival rate than GPC3-negative individuals (54.5% versus 87.7%; P=0.031).[15] These results suggest that GPC3 might be a promising target for cancer immunotherapy.

Biological aspects of GPC3

General considerations

Glypicans are a family of heparan sulfate proteoglycans. To date, six glypicans have been identified (GPC1 to GPC6) in mammals, and two orthologs of the mammalian genes were identified in *Drosophila melanogaster* (Dally- and Dally-like).[8,21] Glypicans of all species are classified into two subfamilies according to their sequence homology.[21] In general, the function of glypicans is to regulate morphogenesis during embryonic development,[22] and mutations cause the overgrowth genetic disease Simpson–Golabi–Behmel syndrome.[23] Several recent studies have revealed that GPC3 is overexpressed in many cancers.

Structure and function of GPC3

GPC3 is a 580-amino acid protein (~60 kDa) that is encoded by nine exons on chromosome X (Xq26). Alternative splicing results in four variants that were isolated from the HepG2 cell line. Fourteen cysteine residues located in the core region are well conserved among glypicans, and contribute to the formation of a unique ternary structure via disulfide bonds. The amino-terminus contains a signal peptide sequence (residues 1–24), which is required for targeting to the cell surface. The carboxyl-terminus contains a hydrophobic region that is associated with the lipid bilayer of the Golgi apparatus. During the transport of GPC3 to the cell surface, the hydrophobic region is truncated by transamidase, and then covalently attached to a GPI anchor via the C-terminus of serine 560.[24] Therefore, the attachment of a GPI anchor is a key post-translational modification that regulates the cellular localization of GPC3.

GPC3 regulates both stimulatory and inhibitory signals through the binding of heparan sulfate chains to signaling molecules such as Wnt, Hedgehog, fibroblast growth factors, bone morphogenetic proteins.[25–31] The core protein also plays an important role for regulating the activity in Wnt and Hedgehog signaling.[27,28,32] Structural information regarding GPC3 is needed to understand these signaling mechanisms, but the three-dimensional structure of GPC3 is yet to be elucidated. Nevertheless, the crystal structure of *Drosophila* Dlp, an ortholog of the mammalian gene, is available.[33] Structural analysis of the Dlp core region revealed an elongated conformation with α-helix packing: this is a unique structure when compared with other proteins. Further structural studies of glypicans are necessary to understand their complex and multifunctional signaling pathways and their regulation of cancer cell growth.

GPC3 biology and disease

GPC3 is expressed in many embryonic tissues in addition to fetal liver and placenta.[34] The overexpression of GPC3 is observed in liver cancer, ovarian cancer, lung cancer, malig-

nant melanoma, and embryonal cancers such as neuroblastoma medulloblastoma and Wilms' tumor.[35–41] Capurro et al demonstrated that the binding of GPC3 to Wnt and Hedgehog activates signaling pathways that promote the growth of HCC cells.[27,28] Moreover, the knockdown of GPC3 using small interfering RNA and subsequent gene expression analysis revealed that suppressing GPC3 inhibited the transforming growth factor-β (TGF-β) receptor pathway and the subsequent growth of HCC cell lines.[42] These suggest that GPC3 is an important target for cancer therapy.[43,44]

It is noteworthy that GPC is a novel serological cancer marker.[12,45,46] Secreted circulating GPC3 is detected in the blood of cancer patients with HCC[11,45] and melanoma,[37,47]and the presence of soluble GPC3 correlates with cancer progression. However, because GPC3 is initially membrane-bound via a GPI anchor, it is currently unknown how GPC3 is secreted into the circulation. It was reported that GPC3 can be cleaved by Notum (α/β-hydrolase enzyme) and furin-like convertase,[48,49] releasing the N-terminal domain and full-length GPC3 from the cell surface.[50,51] Secreted GPC3 might be useful for cancer diagnosis.

GPC3 as a diagnostic marker for HCC

GPC3 expression in HCC at the messenger RNA or protein level

Several studies have suggested that GPC3 is a potential therapeutic target in liver cancer because it is overexpressed in HCC, but is not expressed or is expressed at only low levels in normal adult tissue.[52–54] Hsu et al performed pioneering work to identify GPC3 as a potential biomarker for HCC.[55] When GPC3 was compared with AFP, another established HCC marker, data revealed higher *GPC3* messenger RNA expression compared with serum α-fetoprotein (AFP), levels (71.7% versus 51.3%) based on the analysis of 113 patients with unicentric primary HCC. The authors also reported previously that *GPC3* is specifically overexpressed in HCC by analyzing complementary DNA microarrays containing 23,040 genes. The expression profiles of 20 HCC samples, corresponding noncancerous liver tissues, and various normal human tissues revealed that GPC3 was overexpressed specifically in HCC.[11]

Capurro et al confirmed increased GPC3 expression in HCC patients using a mouse monoclonal antibody (1G12) against a GPC3 C-terminal peptide.[12] Immunohistochemistry revealed that GPC3 was overexpressed in 72% of HCC samples. Therefore, GPC3 might also be useful as an ancillary tool during histopathological diagnostic processes to distinguish HCC from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules.[56]

GPC3 as a serum marker for HCC

Several studies have been performed to validate the diagnostic potential of GPC3 as a serum marker by developing methodologies such as enzyme-linked immunosorbent assays and radioimmunoassays.[45,57] Several antibody-based immunoassays have been developed to assess potential serum biomarkers. Using multiple serum markers, including AFP and protein induced by vitamin K absence or antagonists-II (PIVKA-II), might increase diagnostic accuracy. Although GPC3 is a cell-surface marker, it can be released into the serum by the lipase Notum, which cleaves the GPI anchor.[49] Specifically, Hippo et al reported that GPC3 is cleaved between Arg358 and Ser359, and that the N-terminal fragment of GPC3 is also released into circulation. They reported the usefulness of the N-terminal fragment of GPC3 for diagnosing early-stage HCC.[51] Therefore, GPC3 also exhibits diagnostic value as a serum marker.[57,58] Qiao et al compared the serum levels of three markers (GPC3, human cervical cancer oncogene [HCCR], and AFP) for diagnosing HCC in 189 patients (101 HCC, 40 cirrhosis, and 18 hepatitis cases and 30 healthy control donors). They reported that GPC3 was the most accurate diagnostic marker: using a cutoff of 26.8 ng/mL for the diagnosis of HCC, GPC3 had a sensitivity of 51.5% and a specificity of 92.8%. In addition, the simultaneous detection of three markers increased the sensitivity significantly to 80.2% higher than AFP alone.[58] In a meta-analysis comparing AFP and GPC3 as serum markers for HCC, the pooled sensitivities for AFP and GPC3 were 51.9% and 59.2%, and the pooled specificities were 94% and 84.8%, respectively.[59] This suggests that GPC3 and AFP are comparable serum markers. Serum GPC3 might be a useful tumor marker in patients with HCC. However, the biochemistry of serum GPC3 is yet to be elucidated, and so further studies are needed.

GPC3 as an immunotherapeutic target in HCC

Identification of human leukocyte antigen (HLA)-A2- or A24-restricted GPC3-derived epitope peptides

Identifying TAA-derived epitope peptides is the first step in the development of peptide vaccines. *HLA-A24* is the most common HLA class I allele in the Japanese population (60%).[60,61] Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H-2K^d are similar,[62,63] and the amino acid sequences of human and mouse GPC3 have 95% homology. These studies identified the mouse GPC3-derived

and K^d- restricted cytotoxic T-lymphocyte (CTL) epitope peptide $GPC3_{298-306}$ (EYILSLEEL) in BALB/c mice. This peptide-specific CTL showed specific cytotoxicity against GPC3-expressing or peptide-pulsed cancer cell lines, suggesting that GPC3 was highly immunogenic and could elicit effective antitumor immunity in mice. Importantly, there was no evidence of autoimmune reactions in the treated mice.[64] Because of the similarities in the peptide binding motifs between H-$2K^d$ and HLA-A24, this peptide was applicable for immunotherapy in HLA-A24-positive patients.

HLA-A2 is also expressed in 40% of Japanese individuals, as well as other ethnic populations.[60,65] An HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide was also identified using *HLA-A2.1* transgenic mice.[58] A binding assay was performed, and it was reported that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could bind to HLA-A*02:06 and HLA-A*02:07. This suggests that HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) might be effective in HLA-A*02:06 and HLA-A*02:07 patients.

These GPC3-derived peptide-specific CTLs could be induced from the peripheral blood mononuclear cells of HCC patients by in vitro stimulation with peptide. The adoptive transfer of these GPC3-derived peptide-specific CTLs reduced the mass of human HCC tumors implanted into non-obese diabetic/severe combined immunodeficiency mice.[66]

GPC3-targeted vaccine therapy

The authors recently completed an investigator-initiated Phase I clinical trial of GPC3-derived peptide vaccines to evaluate their safety, tolerability, and efficacy in patients with advanced HCC.[67] Thirty-three advanced HCC patients were enrolled and received escalating doses of GPC3-derived peptide vaccine (0.3, 1.0, 3.0, 10, and 30 mg/patient). On days 1, 15, and 29, peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant by intradermal injection. $GPC3_{298-306}$ (EYILSLEEL) peptide was used in 17 HLA-A24-positive patients, and $GPC3_{144-152}$ (FVGEFFTDV) peptide was used in 16 HLA-A2-positive patients.

Dose-limiting toxicity and dose-specific adverse events were not seen, and GPC3-derived peptide vaccine treatment was well tolerated. One of the thirty-three patients was judged to have a partial response, whereas 19 patients exhibited stable disease after 2 months according to Response Evaluation Criteria In Solid Tumors (RECIST).[68] The disease control rate (partial response plus stable disease) was 60.6% after 2 months. The median time to tumor progression was 3.4 months (95% confidence interval [CI] 2.1–4.6), and the median overall survival was 9.0 months (95% CI 8.0–10.0).

Immunologically, the frequency of GPC3-peptide-specific CTL in the peripheral blood correlated with the overall survival of HCC patients. In the multivariate analysis, GPC3 peptide-specific CTL frequency was a predictive factor for overall survival. The median overall survival of all 33 patients was 12.2 months (95% CI 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs compared with 8.5 months (95% CI 3.7–13.1) in individuals with a low frequency (P=0.033). Moreover, the infiltration of cluster of differentiation (CD)8-positive T-cells into HCC cells was confirmed.

Based on this Phase I study, a Phase II study of the GPC3-derived peptide vaccine is ongoing in an adjuvant setting (UMIN-CTR: 000002614). Forty-four patients with HCC who had undergone surgery or radiofrequency ablation were enrolled. The primary end points of this study were the 1- and 2-year recurrence rates, and the secondary end point was the immunological response. Patient enrollment has been completed, and the study is ongoing. An additional sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway.

Anti-GPC3 antibody therapy

GPC3 has been suggested as a potential target for antibody-based therapy in liver cancer because of its high-level expression in HCC. The murine monoclonal antibody GC33, which binds specifically to the C-terminal region of GPC3 with a high affinity, caused significant antibody-dependent cellular cytotoxicity against HCC cells, and exhibited potent antitumor activity in xenograft models.[69–72] For the clinical application of GC33, a humanized GC33 was generated using complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. To improve the stability of the humanized GC33, it was further optimized by replacing the amino acid residues that might affect the structure of the variable region of its heavy chain.[73]

Because of these preclinical data highlighting the relevance of GPC3 as a potential therapeutic target in HCC, a first-in-man Phase I clinical trial to assess the safety, tolerability, and pharmacokinetics of GC33 in patients with advanced HCC was performed.[74] A total of 20 patients were enrolled, and were assigned to receive GC33 at one of four sequentially increasing dose levels (2.5, 5, 10, and 20 mg/kg) weekly by intravenous infusion. The tumor expression of GPC3 was examined in biopsied specimens using immunohistochemical staining. A total of 56% of the patients had a high total GPC3-staining score. This study provided the

initial clinical data regarding the safety profile and pharmacokinetic features of GC33, and revealed potential antitumor activity that might be associated with the expression of GPC3 in tumors. Stable disease was seen in four patients, all of whom exhibited high GPC3 expression. The median time to progression was significantly longer in patients with tumors expressing high levels of GPC3 than in patients with low GPC3 expression.

GC33 is now being assessed in Phase II clinical trials in second-line HCC patients who have progressed after one line of systemic therapy and whose tumors exhibit positive GPC3 immunohistochemical staining (NCT01507168). Additional antibodies that target GPC3 for HCC treatment, human (MDX-1414 and HN3) and humanized mouse (YP7) antibodies, are at different stages of preclinical development.[75] These trials will define the potential of GPC3 as a novel antibody therapy.

Potential of GPC3 for other cancers

GPC3 is also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian clear-cell carcinoma (CCC), and lung squamous cell carcinoma.[37-41] However, Kim et al reported that GPC3 is downregulated in lung cancer. Thus, the overexpression of GPC3 in lung cancer is controversial.[76] GPC3 has been investigated in some of these tumors as a potential immunotherapeutic target or diagnostic marker.

Melanoma

GPC3 messenger RNA and protein was identified in >80% of melanoma and melanocytic nevus patients.[39] In the authors' previous study, GPC3 protein was detected in the sera of 39.6% melanoma patients, but not in healthy donors. The positive detection of serum GPC3 was significantly higher than that of 5-S-cysteinyldopa and melanoma-inhibitory activity, both of which are well-known tumor markers for melanoma. Surprisingly, GPC3 could be detected even in patients with stage 0 in situ melanoma.[37] The combination of secreted protein acidic and rich in cysteine (SPARC) and GPC3 was also a useful tumor marker for melanoma: 66.2% of melanoma patients at stages 0–II exhibited positive SPARC or GPC3 expression.[47] This suggests that GPC3 is a novel tumor marker that is useful for the diagnosis of melanoma, particularly during the early stages.

Ovarian carcinoma

Ovarian CCC is the second most common epithelial ovarian carcinoma subtype in Japan. Ovarian CCC is associated with a poor prognosis and increased chemoresistance compared with other epithelial ovarian carcinoma subtypes.[77,78] GPC3 was expressed in ~40% of CCC patients, and there was a tendency toward poor progression-free survival in GPC3-positive patients at stage I.[79] GPC3 expression was responsible for CTL recognition, and subtoxic dose chemotherapy made tumor cells more susceptible to the cytotoxic effects of CTL.[80] A Phase II trial of a GPC3-derived peptide vaccine in ovarian CCC patients is ongoing (UMIN-CTR: 000003696), and some chemotherapy-refractory ovarian CCC patients have achieved a significant clinical response.[81]

Pediatric tumors

A Phase I trial using a GPC3-derived peptide vaccine for pediatric patients with hepatoblastoma, nephroblastoma, or yolk sac tumors is ongoing (UMIN-CTR: 000006357). The safety and optimal dose of GPC3 peptide vaccines for pediatric cancer patients has not yet been reported.

Conclusion

Although immunotherapy is a potentially attractive treatment modality, its antitumor effects in advanced HCC are not dramatic. GPC3 is overexpressed in HCC but its expression in most adult normal tissues is low. GPC3 expression is correlated with poor prognosis in HCC, suggesting it to be an ideal tumor antigen. GPC3 is thought to play a role in regulating cancer cell growth, although our structural and biological knowledge of GPC3 remain limited. Recent studies have shown the utility of GPC3 as a serum and immunohistochemical marker for the diagnosis of HCC. In addition, although studies assessing GPC3-targeted immunotherapies against HCC (such as vaccine and antibody therapies) have shown good safety and tolerability, sufficient clinical effects have not yet been observed. Further analysis and knowledge of GPC3 biology and its potential as an immunotherapeutic target are needed to allow the development of more effective GPC3-targeted cancer therapies. Although current GPC3-targeted immunotherapies for HCC are in the preclinical and clinical trial phases of development, they are expected to yield clinical success in the near future.

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Disclosure

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Original Article

Parents' perception of pediatric cancer centers in Japan

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Abstract ***Background***: In Japan, more than 160 hospitals provide care for approximately 2500 pediatric patients diagnosed with cancer each year. Not all hospitals, however, are fully capable of providing state-of-the-art care due to a lack of experienced personnel or up-to-date facilities. The aim of this study was to solicit parents' experiences during their children's cancer treatment and opinions about the centralization of medical resources to core pediatric cancer centers.
Methods: A structured questionnaire was sent to parents of children who had received cancer treatment.
Results: Eighty-two questionnaires were completed and analyzed. Parents reported a need for improved psychological support for their children and family members as well as accommodation for families during cancer therapy. Most parents had positive opinions about the centralization of medical resources to core centers but were concerned about the accessibility of the centers and increasing burdens placed on families living in remote areas.
Conclusion: The demand for psychological care for families during children's cancer treatment is highlighted. Improved accommodation and greater financial and social support for families living in remote areas should be preconditions for the future centralization of core pediatric cancer centers.

Key words cancer care facility, caregiver, health facility environment, health resources, health services accessibility.

It is important to provide children with a comprehensive, multidisciplinary approach to cancer treatment. Superior outcomes of cancer treatment are observed when children receive up-to-date diagnostic, supportive, and specific care by a team of specialists at pediatric cancer centers.[1] In Japan, there are approximately 2500 newly diagnosed cases of pediatric cancer per year, with medical care provided by more than 160 hospitals. A major concern is that many hospitals treat only a handful of new cases a year and may not be capable of providing children with state-of-the-art care due to a lack of experienced and committed personnel or fully equipped facilities. To improve pediatric oncologic care in Japan, therefore, the reallocation and concentration of medical resources to core centers seems inevitable.

As part of a larger effort to develop guidelines for core pediatric cancer centers to improve oncologic care for children, we conducted a survey of parents whose children received cancer treatment. The aim was to highlight parents' experiences during their children's cancer treatment and to understand their perceptions of the centralization of medical resources to core pediatric cancer centers.

Methods

Participants were parents whose children received cancer treatment. At the end of 2009, the study was advertised in a newsletter issued by Gan-no-kodomo-wo-mamoru-kai, a nationwide non-profit organization consisting of parents whose children have cancer, which aims to support children, their family members, and researchers in the field of pediatric oncology. A structured questionnaire was sent to parents who contacted the study coordinator and expressed their willingness to participate in the study. Parents were asked to return the completed questionnaire via mail. Analysis of the returned questionnaires was conducted anonymously. The study was approved by the ethics board of Gan-no-kodomo-wo-mamoru-kai.

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Results

Family characteristics

In February 2010, the questionnaires were sent to 103 parents whose children received cancer treatment. Of the 82 questionnaires that were completed and returned, 73 were completed by the mother, two were completed by the father, and seven were completed by both the mother and father. Characteristics of children's cancer diagnoses are listed in Table 1. Of the 82 families, 40 children (48.8%) were treated at university hospitals and 22 (26.8%) at national cancer centers or children's hospitals. The remaining 20 children (24.4%) were treated at local community hospitals. During the children's cancer treatment, 40 families (48.8%) lived in the Kanto area, including 20 families in Tokyo. At the time of cancer diagnosis, 61 children (73.4%) had at least one sibling, and three children had a single parent.