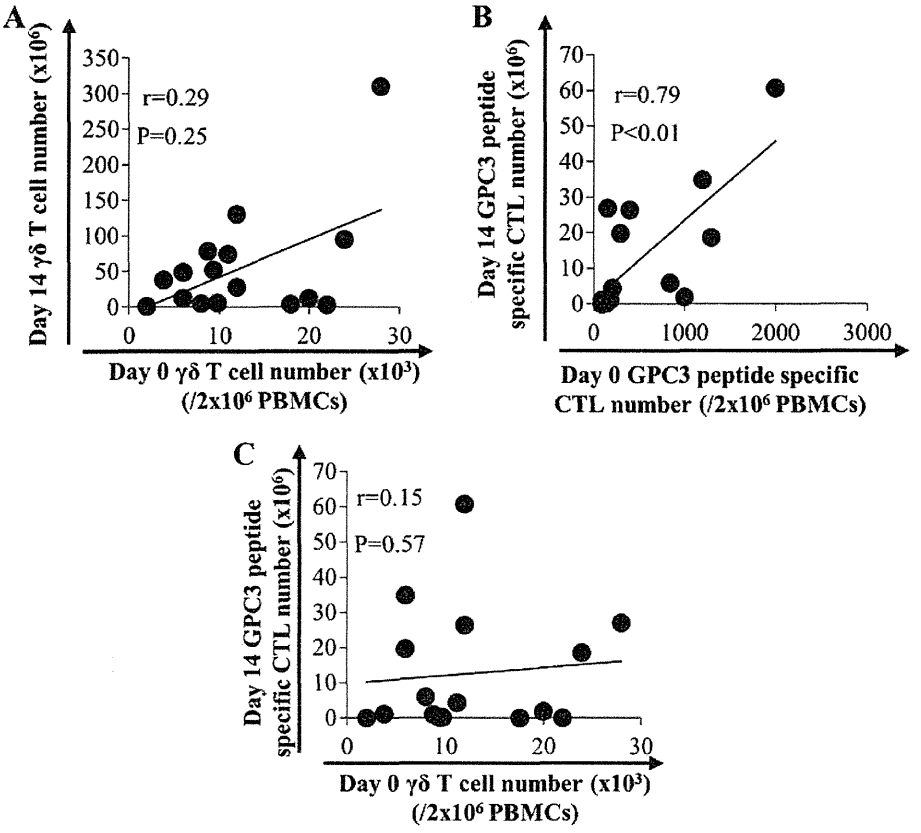

Figure 2. Efficacy of the method to induce expansion of GPC3 peptide-specific CTLs. (A) The correlation between the number of γδ T cells before and after expansion (n=16). (B) The correlation between the number of GPC3 peptide-specific CTLs before and after expansion. The number of GPC3 peptide-specific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

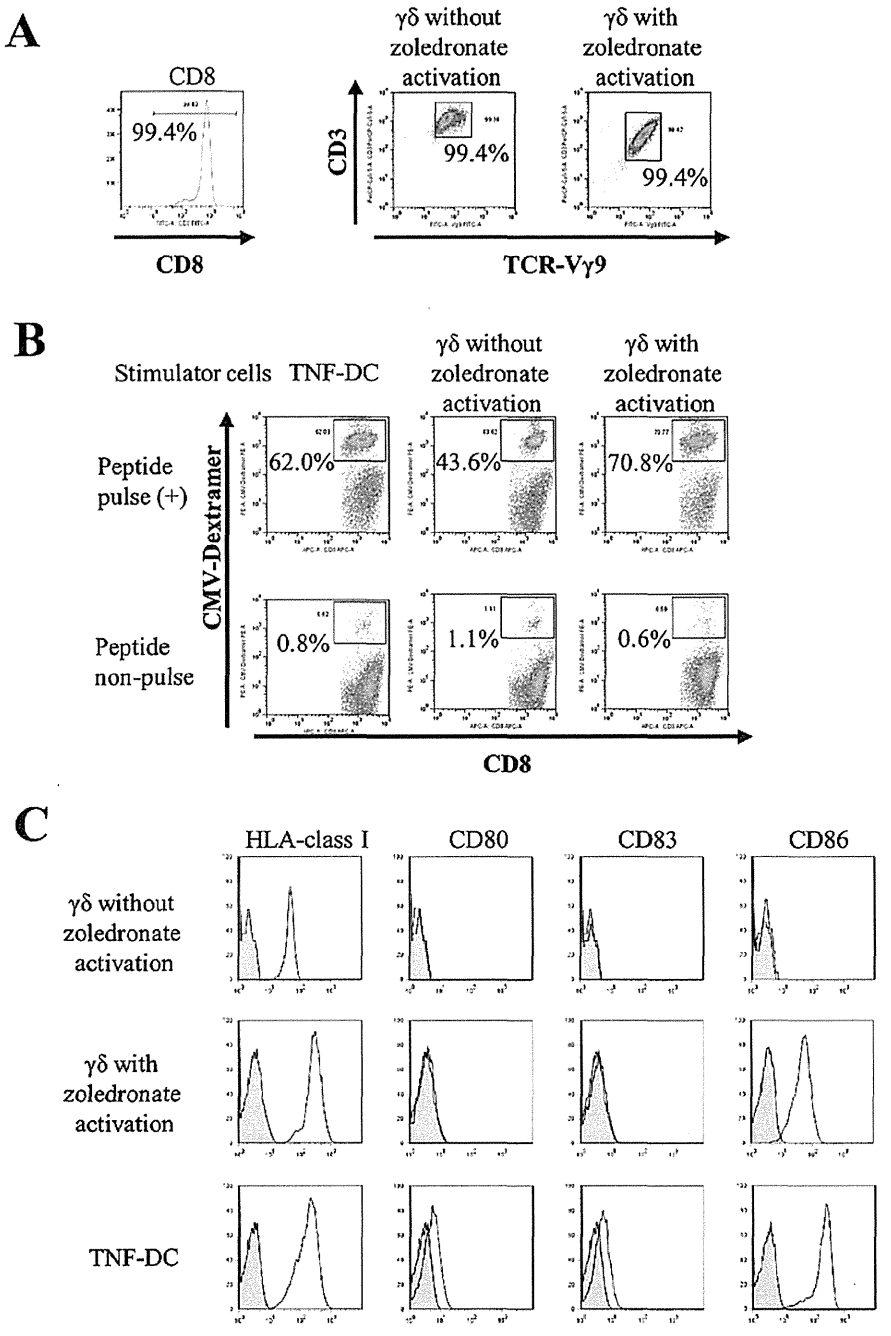

Figure 3. Activated γδ T cells function as antigen-presenting cells. (A) The percentages of sorted cells were analyzed using flow cytometry. The purity of sorted $CD8^+$ cells, γδ T cells without zoledronate activation and γδ T cells with zoledronate activation were greater than 99%. (B) The responder $CD8^+$ 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-

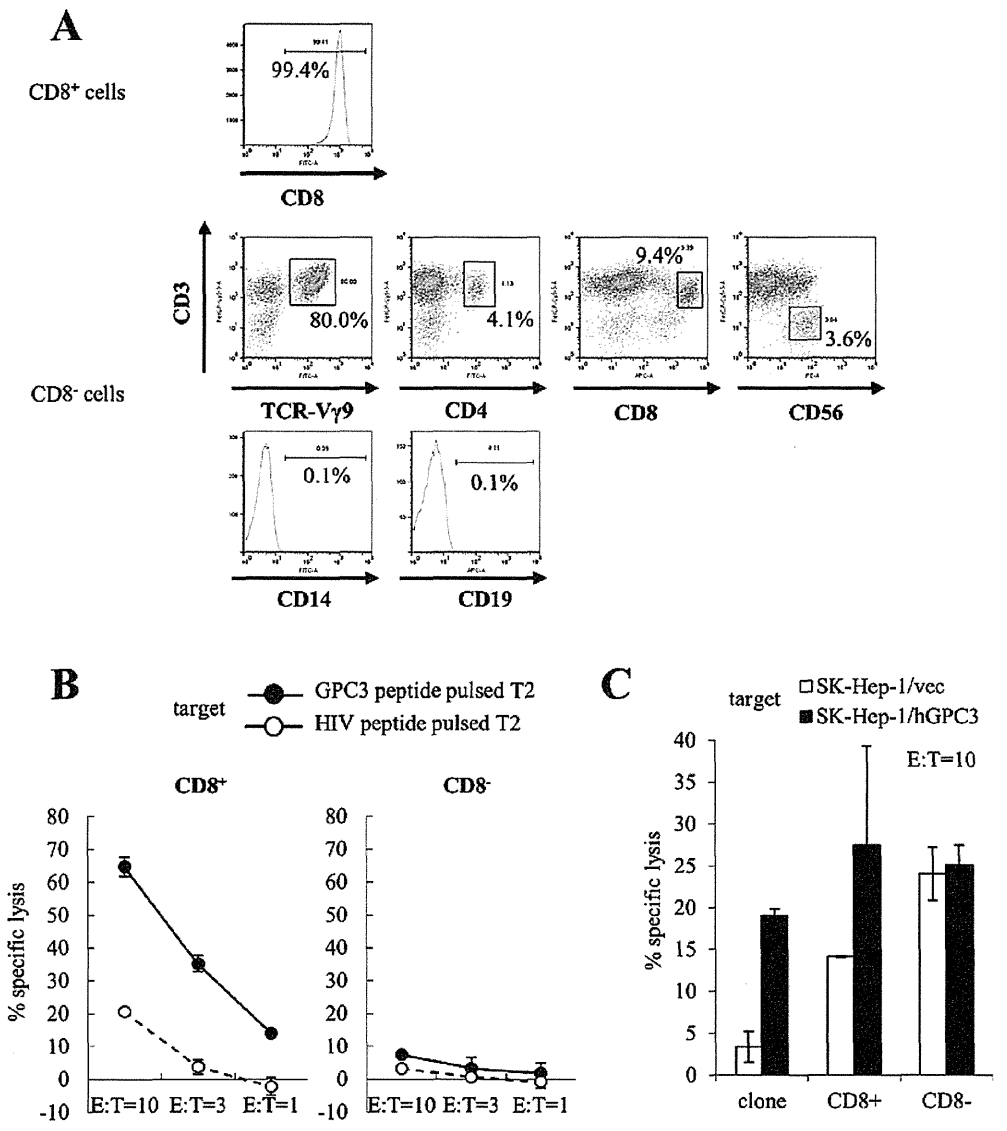

Figure 4. Cytotoxicity assay of cultured cells. We used CD8+ and CD8- cells that were isolated from cultured cells using CD8 microbeads at day 14 as effector cells. A GPC3 peptide-specific CTL clone was used as a positive control. We performed cytotoxicity assays using expanded cells from four patients. 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.

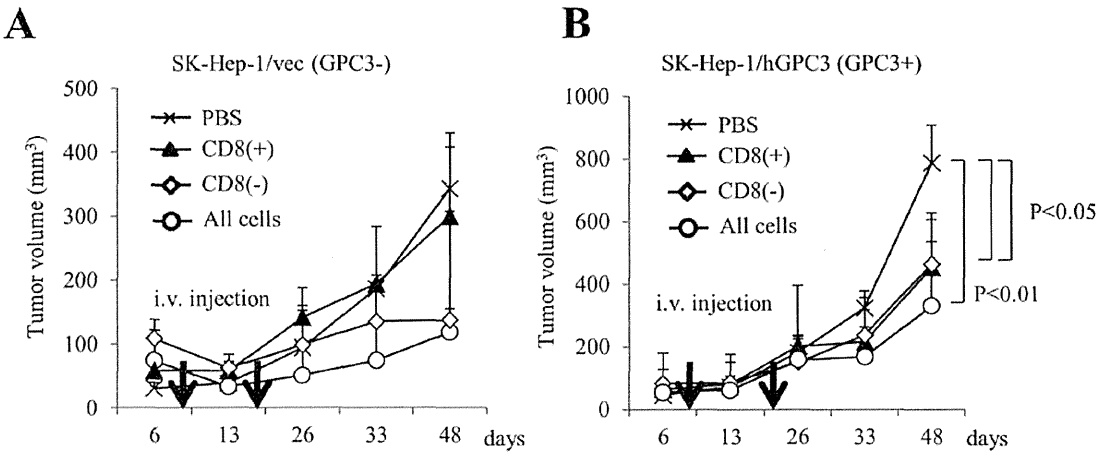

Figure 5. Antitumor activity of γδ T cells and GPC3-specific CTLs *in vivo*. We subcutaneously inoculated (A) SK-Hep-1/vec or (B) SK-Hep-1/hGPC3 cells into NOD/SCID mice and intravenously injected effector cells. We performed adoptive cell transfer of expanded cells using five mice per group. Data represent 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.

Acknowledgements

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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.

Table 1 Characteristics of children's cancer diagnoses

Sex (n = 81)	n (%)
Male	44 (54.3)
Age at diagnosis (n = 81)	
<12 months	9 (11.1)
1–5 years	36 (44.4)
6–15 years	30 (37.0)
>15 years	6 (7.4)
Year of diagnosis (n = 82)	
1970–1979	2 (2.4)
1980–1989	3 (3.7)
1990–1999	10 (12.2)
2000–2009	67 (81.7)
Diagnosis (n = 67)	
Leukemia/lymphoma	27 (40.3)
Solid tumor	15 (22.4)
Brain tumor	25 (37.3)
Current status (n = 82)	
Cancer treatment ongoing	9 (11.0)
Cancer treatment completed	47 (57.3)
Died	26 (31.7)

Health-care personnel and availability of facilities

Parents were asked to select health-care personnel involved in the care during the cancer treatment and facilities available at the hospitals from the list provided as in Table 2. Overall, certified child care staff were the health-care personnel who were most frequently involved, followed by social workers, pharmacists, and physiotherapists/occupational therapists. One-third of parents in community hospitals reported that apart from doctors and nurses, no health-care personnel were involved in their children's care.

Laundry and cafeteria facilities were available to most families. Housing for families, including in-hospital rooms for overnight stays or facilities adjacent to hospitals were available for two-thirds of families in children's hospitals compared to only 15% of families in university hospitals and community hospitals.

Apart from the facilities listed in Table 2, parents were asked to indicate what other types of facilities were useful or would have been useful. A place where children's siblings could stay during parents' hospital visits (n = 9) and a place where families could spend time with children or communicate with other families (n = 9) were most frequently reported as facilities that would have been useful. Also, a drug store was frequently reported as a facility that was useful (n = 7) or that would have been useful (n = 3).

Aspects of the cancer treatment environment that need improvement

The question was open-ended, and asked "what aspects of the cancer treatment or treatment environment need improvement". Sixty-three parents responded to this question, and their responses were placed into the six categories shown in Figure 1. The two most common responses were related to children's quality of life during treatment: psychological support (n = 21) and facilities and equipment for study and play (n = 16). The two next most common responses were related to family needs: support and care for families (n = 16) and accommodation (n = 11). Other responses not shown in Figure 1 included support for children's siblings (n = 3), better coordination with subspecialty consultants (n = 3), and after-care follow up (n = 2).

Perceptions about core pediatric cancer centers

After parents were told that "cancer treatment for children requires a multidisciplinary team of personnel and well-equipped facilities; given the rarity of pediatric cancer and the limited availability of resources, the concentration of medical resources to core pediatric cancer centers will be required to improve oncologic care for children", they were asked for their opinion regarding the centralization of pediatric cancer centers. Of the 38 parents who shared their opinions, 23 (60.5%) were supportive of

Table 2 Health-care personnel and availability of facilities

	Total (n = 82) n (%)	Children's hospital (n = 22) n (%)	University hospital (n = 40) n (%)	Community hospital (n = 20) n (%)
Health-care personnel involved in care				
Certified child care staff	32 (39.0)	14 (63.6)	13 (32.5)	5 (25.0)
Social worker	31 (37.8)	10 (45.5)	16 (40.0)	5 (25.0)
Pharmacist	27 (32.9)	3 (13.6)	21 (52.5)	3 (15.0)
Physiotherapist/Occupational therapist	23 (28.0)	7 (31.8)	11 (27.5)	5 (25.0)
In-hospital school teacher	20 (24.4)	9 (40.9)	10 (25.0)	1 (5.0)
Clinical psychologist	12 (14.6)	3 (13.6)	8 (20.0)	1 (5.0)
Palliative care team	6 (7.3)	2 (9.1)	4 (10.0)	0
Nutrition support team	5 (6.1)	0	5 (12.5)	0
Child life specialist	5 (6.1)	2 (9.1)	3 (7.5)	0
None apart from doctors and nurses	13 (15.9)	2 (9.1)	5 (12.5)	6 (30)
Available hospital facilities				
Laundry	52 (63.4)	11 (50.0)	28 (70.0)	13 (65.0)
Cafeteria	56 (68.3)	16 (72.7)	28 (70.0)	12 (60.0)
Kitchen	15 (18.3)	4 (18.2)	10 (25.0)	1 (5.0)
Bath or shower room	46 (56.1)	10 (45.5)	27 (67.5)	9 (45.0)
Accommodation	23 (28.0)	14 (63.6)	6 (15.0)	3 (15.0)

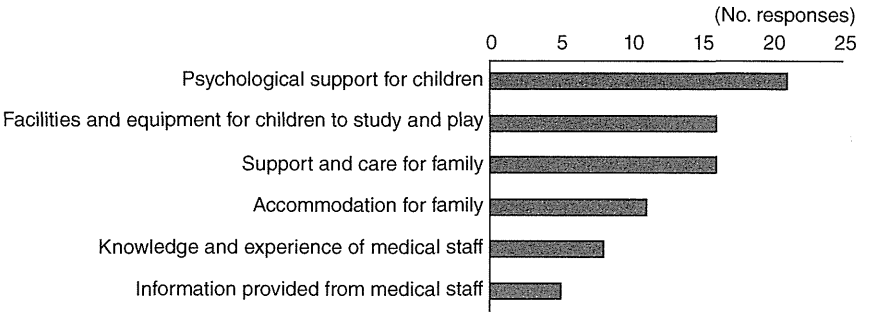

Fig. 1 Aspects of the cancer treatment environment that need improvement.

the principle of centralization with the expectation that cancer treatment would be improved. Four (10.5%) reported that they would be supportive under the conditions that there would be a sufficient number of centers easily accessible by everyone ($n = 2$) or that accommodation and other travel support would be available for families living in remote locations ($n = 2$). Eleven parents (28.9%) were reluctant about centralization because this would increase the burden on families, especially those living in remote areas. Of these 11 parents, five also noted that although they understand the importance of centralization, it would be impossible for families to travel further for treatment based on their own experience.

Travel time to the hospital

As shown in Figure 2, most parents reported that they lived within 1 h from the hospital where their children received cancer treatment, whereas eight (10.3%) reported that the hospital was >2 h away. With regard to future centralization of pediatric cancer centers, 72.7% of parents considered that a travel time >1 h each way was not acceptable for hospitalization, and a travel time >2 h was acceptable to only 3.9% of parents. For outpatient clinic visits, 30.8% of parents considered that a travel time >30 min each way was not acceptable, and a travel time >1 h was acceptable to only 14.1% of parents.

Discussion

In 2011, the Japanese Society of Pediatric Hematology/Oncology started registering institutions that meet basic requirements for providing oncologic care for pediatric patients and educational programs for pediatricians. As of October 2012, 89 institutions were registered.[2] A certification program for pediatric oncologists trained at the registered institutions will soon begin. Seven Japanese prefectures, however, possess no registered institutions, and the appropriate centralization and allocation of medical resources among the registered institutions remains a challenge. In the present study, we inquired about parents' experiences during their children's cancer treatment and sought their opinion regarding the future centralization and allocation of medical resources. We believe that parents' perceptions and opinions deserve special attention and are worth considering, given that they endure substantial personal difficulties during their children's cancer treatment.

We found that clinical psychologists were involved in care for only 14.6% of families, and this small percentage may be reflected by the low parental satisfaction with psychological care for their children during cancer treatment. It should also be noted that almost one-fourth of parents reported that an improvement in care for family members was needed. More than a few parents specifically commented on the need for psychological care for children's siblings. Furthermore, involvement of a palliative care team was reported by only six parents despite the fact that 26 children in the study had died. A project funded by the Ministry of Health, Labour and Welfare was recently launched to develop a pediatric palliative care education program for health-care professionals,[3] which will hopefully improve palliative care for pediatric patients in the near feature.

Regarding the centralization of medical resources to core pediatric centers, this study clearly demonstrates that the main concern of parents is geographical proximity to the hospital.

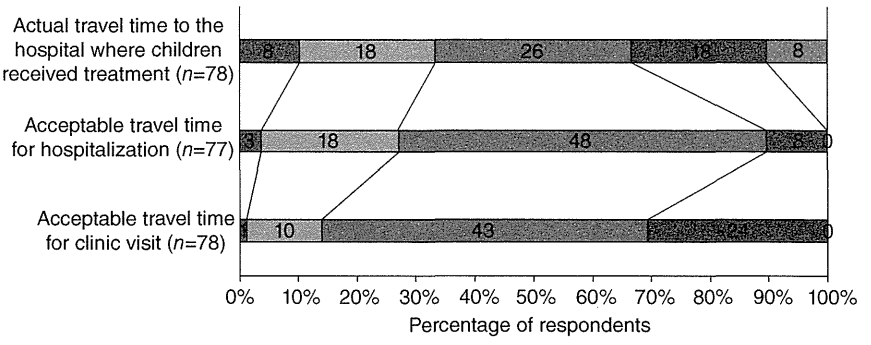

Fig. 2 Travel time to the hospital. (■) >2 h; (■) >1 h; (■) ≤1 h; (■) <30 min; (■) <15 min.

Although 10.3% of parents reported that their commute to the hospital was >2 h, only 3.9% and 1.3% considered that a commute >2 h is acceptable for hospitalization or clinic visits, respectively. Accessibility to the hospital for cancer treatment has a great impact on the lives of families. Improved accommodation and enhanced financial and social support for families living in remote areas should be preconditions for the future centralization of core pediatric cancer centers.

The main limitation of the present study is its generalizability. Participating parents were members of the Gan-no-kodomo-wo-mamoru-kai community and were interested in sharing their experience and opinions. Parents who did not participate in the study may have had different experiences and opinions. Also, because the study included parents whose children were diagnosed in the 1980s and 1990s, some responses may not reflect the current situation. Data collected in this study did not contain the detailed information about the disease course. Parents' experience as well as the need for health-care personnel and hospital facilities would vary depending on multiple factors in the disease course such as severity of disease symptoms or treatment-related adverse reactions, treatment modality and length of the treatment.

In conclusion, the present survey of parents whose children received cancer treatment has identified a demand for psychological care for children and their families during cancer therapy. Although the concept of centralization of medical resources was generally accepted by parents with an expectation of better cancer treatment, serious consideration should be given to the accessibility of cancer centers and the burden placed on families living in remote areas.

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Original contribution

Close correlation between CXCR4 and VEGF expression and frequent CXCR7 expression in rhabdomyosarcoma☆,☆☆

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Summary CXC chemokine receptor 4 (CXCR4) expression is reportedly correlated with both vascular endothelial growth factor (VEGF) expression and poor prognosis in a variety of cancers. Its relation to CXC chemokine receptor 7 (CXCR7) is also noted in several malignancies, including rhabdomyosarcoma (RMS) cell lines. However, the correlations between these chemokine receptors and angiogenic factors have not yet been adequately investigated in RMS clinical specimens. By immunohistochemistry, we assessed CXCR4, CXCR7, CC chemokine receptor 6, CC chemokine receptor 7, VEGF expression, microvessel density, and MIB-1 labeling index in 82 formalin-fixed RMS specimens, including 34 primary alveolar RMS and 44 primary embryonal RMS (ERMS). Twenty-six frozen samples were available for investigation by quantitative reverse transcription polymerase chain reaction to detect the messenger RNA expression levels of these molecules. We also evaluated their significance with respect to clinicopathological factors and patient survival rates. Primary RMS showed high expression of CXCR7 (83.1%) regardless of the histologic subtype. High cytoplasmic CXCR4 and high VEGF expression revealed significant correlations in both ERMS and alveolar RMS ($P = .0051$ and $P = .0003$, respectively). By univariate analysis of ERMS cases, the tumors with high VEGF expression showed significantly poor prognoses ($P = .0017$). High VEGF expression also was the independent adverse prognostic factor for ERMS. Because CXCR4, CXCR7, and VEGF are widely expressed in RMS, the combination of these antagonists may provide a potential target for molecular therapy.

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1. Introduction

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue sarcoma in childhood and adolescence. There are 2 major histologic subtypes in RMS: alveolar RMS (ARMS) and embryonal RMS (ERMS). The former is well known to be associated with *PAX3-FKHR* or *PAX7-FKHR* gene fusions and with significantly poor prognosis. Meanwhile, no specific fusion gene has been identified in ERMS [1].

Many kinds of chemokines display various roles in immunity, regulating angiogenesis, promoting the proliferation of tumor cells, and mediating organ-specific metastases [2]. Chemokine receptors, especially CXC chemokine receptor 4 (CXCR4) [3-7] and CXC chemokine receptor 7 (CXCR7) [8,9], have been suggested to play an important role in metastatic behavior to specific target organs [10]. CC chemokine receptor 7 (CCR7) is primarily responsible for lymph node metastases [11,12], whereas CC chemokine receptor 6 (CCR6) is suggested to have relation to liver metastases [13] and tumor progression [14].

Vascular endothelial growth factor (VEGF), known as a critical mediator of angiogenesis and tumor proliferation, has revealed its overexpressed status in a variety of cancers [15]. Thus, the therapeutic approaches targeting VEGF have been explored in several cancers [16-18].

CXCR4 has been believed to be the only receptor that binds stromal cell–derived factor (SDF-1). Recently, a new SDF-1 binding receptor, CXCR7, was identified through an experiment that revealed discrepancies between CXCR4 expression and SDF-1 binding on different cell lines, using cells from CXCR4-deficient mice [19].

In RMS cell lines, it has been reported that CXCR4 was expressed at much higher levels by highly metastatic ARMS lines, whereas CXCR7 was expressed in both ARMS and ERMS, with higher expression in ERMS cell lines [20].

A significant correlation between the messenger RNA (mRNA) levels of VEGF and CXCR4 in breast cancer tumor tissue was reported in a preliminary investigation [21]. In addition, the autocrine manner of VEGF's involvement in the CXCR4/SDF-1 pathway in the invasion of breast carcinoma cells has been demonstrated [8]. Furthermore, the CXCR4/SDF-1 system was found to be involved with the PI3K/Akt pathway in a breast cancer cell line [21] and with the ERK pathway in a pancreatic cancer cell line [22].

In the present study, we immunohistochemically evaluated CXCR4, CXCR7, CCR6, CCR7, and VEGF protein expression to investigate these protein expressions in a large series of RMS clinical cases and examined the mRNA expression levels of *CXCR4*, *CXCR7*, *CCR6*, *CCR7*, and *VEGF* in frozen samples using quantitative reverse transcription polymerase chain reaction (RT-PCR). Moreover, we compared these results with clinicopathological parameters, angiogenesis factors, and prognosis in RMS.

There have been no reports on the relationship of metastasis-related chemokine receptors and angiogenesis factors in the aspects of tissue expression in large series of clinical RMS cases. The investigation for these protein expressions in the clinical tumor tissue could aid in the search for new potential therapeutic targets.

2. Materials and methods

2.1. Patients and tissue specimens

Eighty-two paraffin-embedded RMS specimens obtained from 78 patients were collected from the soft tissue tumor file registered between 1976 and 2007 at the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. The 82 specimens from these 78 patients included 78 primary tumors, 3 metastatic tumors (2 in lung and 1 in thigh), and 1 locally recurrent tumor. The histologic diagnosis of RMS and its subtype was confirmed according to the latest World Health Organization classification [1]. The anatomical locations of the primary tumor sites were categorized as favorable or unfavorable tumor sites according to Intergroup Rhabdomyosarcoma Study V (IRS-V) [23]. The patients were classified according to the pretreatment staging system by Intergroup Rhabdomyosarcoma Study Group [23]. Furthermore, 26 of the specimens were also snap frozen in liquid nitrogen at the time of the surgical procedure and stored at −80°C until use. The institutional review board at Kyushu University approved this study (permission code: 25-143).

2.2. Immunohistochemistry

Immunohistochemistry was performed in 82 tumors, using formalin-fixed tissue sections in concordance with frozen material. Sections were cut at widths of 4 μm from paraffin-embedded material, dewaxed with xylene, and rehydrated through a graded series of ethanol. After the inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted.

The following antibodies were used as primary antibody: anti-CXCR4 (12G5, monoclonal, 1:100; BD PharMingen, San Diego, CA), anti-CXCR7 (polyclonal, 1:200; GeneTex, Irvine, CA), anti-CCR6 (11A9, monoclonal, 1:200; BD Pharmingen), anti-CCR7 (polyclonal, 1:250; Capralogics, Hardwick, MA), anti-VEGF (A-20, polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD31 (JC70A, monoclonal, 1:20; Dako, Glostrup, Denmark), and anti–Ki-67 (MIB-1, monoclonal, 1:100; DAKO). For staining with CXCR4, CXCR7, CCR6, CCR7, VEGF, and Ki-67, sections were pretreated with microwave irradiation in citrate buffer or EDTA buffer for antigen retrieval. As for CD31, sections were pretreated with trypsin for 30 minutes.

Sections from human tonsils for CXCR4, CCR6, and CCR7 and sections from human renal cell carcinoma for CXCR7 were used as positive controls [24,25].