たパイを目指してのライバル関係となり，申請先からは小児固形がん研究組織のまとまりのなさを指摘されることにもなる。実際，研究費を獲得しても厚生労働省科学研究費のような公的研究費は原則3年間であるため，臨床試験の結果を期間内に示すことは困難であるし，また，試験の立ち上げに時間がかかるために研究費を効率的に使用することができていない。白血病も含む小児がん研究に対して毎年1億円以上の厚生労働省からの研究費が以前より投入されており，国からも小児がん研究の充実が求められているところである。研究費のほとんどは事務局の維持，データセンターの経費，中央病理診断の経費などに費やされる。今後は戦略的に共同機構内のどこかのグループが研究費の獲得を目指す，あるいは共同機構として研究費を申請することで，これらのすべてのがん種別グループの中央経費を賄うことができる。共同機構全体として研究費を獲得した場合は，全研究グループの活動を研究成果として利用することができる。

4．がん種別研究グループの情報公開が容易となる

これまで各グループは個別に研究会を開催してきたため，多くのグループに参加している施設はこれらすべてに出席することが困難であった。また，参加していない研究グループの情報には接する機会がなかった。共同機構の研究会および総会として，全グループが同時期に活動状況の報告や検討会を行うことで各参加施設の利便性をはかることができる。

5．研究の質の向上と研究の発展

すべての臨床試験のデータマネージメントを中央データセンターで行うことで臨床試験の質の向上が担保される。また，疾患別グループの枠を超えた研究を容易に行うことが可能となるため，研究の発展が望める。具体的には新規薬剤の第Ⅰ相試験などの複数がん種を対象とした早期試験などが考えられる。

6．ナショナルスタディグループとしての国内外へのアピール

国を代表する研究グループとして認められるためには，全国の大半を網羅した組織であること，国の予算により運営されていることなどが必要である。これまでの個々の研究グループを共同機構として大きく包括することで，ナショナルスタディグループとしての資格を有することになり，わが国および世界における位置を確立することができる。また，国の小児がん研究支援の受け皿となることができる。

Ⅴ．共同機構の活動

本特集の別稿「小児固形腫瘍観察研究」で詳細は述べられるが，2011年4月より，小児固形がん観察研究を開始した（図2）。脳腫瘍の一部疾患についてはその半年前に先行開始している。固形腫瘍が発生した場合，疑われる疾患を問わず，すべての症例で中央病理診断と余剰検体の保存を行うというものである。その際，症例登録が行われ，転帰調査も行うことで，正確な診断に基づいた各疾患の予後が明らかとなることが期待される。このシステムは各研究グループの実施している臨床試験で利用可能なものであり，各グループでは新たな研究を開始するのにあわせて，順次移行する予定である。これにより共同機構の目的の多くが達成される。数年後には疫学研究のデータが蓄積されると同時に，保存検体を用いた研究なども活発になってくることが期待される。それに備えてこれらのデータや検体の公平な使用を担保するための研究審査委員会を立ち上げた。委員として各研究グループから1名ずつが推薦され，また，共同機構参加施設以外からの登録も予想されるため，一般施設代表も選ばれている。ちなみに一般施設代表はもっとも登録数の多い施設から選ばれることになっている。

Ⅵ．今後の方向性

全国の小児がん診療施設に共同機構の存在とその意義が浸透し，そのうえで有効活用されることが最優先の課題である。そのためには双方向の情報の伝達が重要であると考えられることから少なくとも年1回は総会などその機会を設けることにしている。また，今後研究を遂行するのに必要な組織，例えばプロトコール作成支援のための委員会，医学統計の部門などの設置も行っていく予

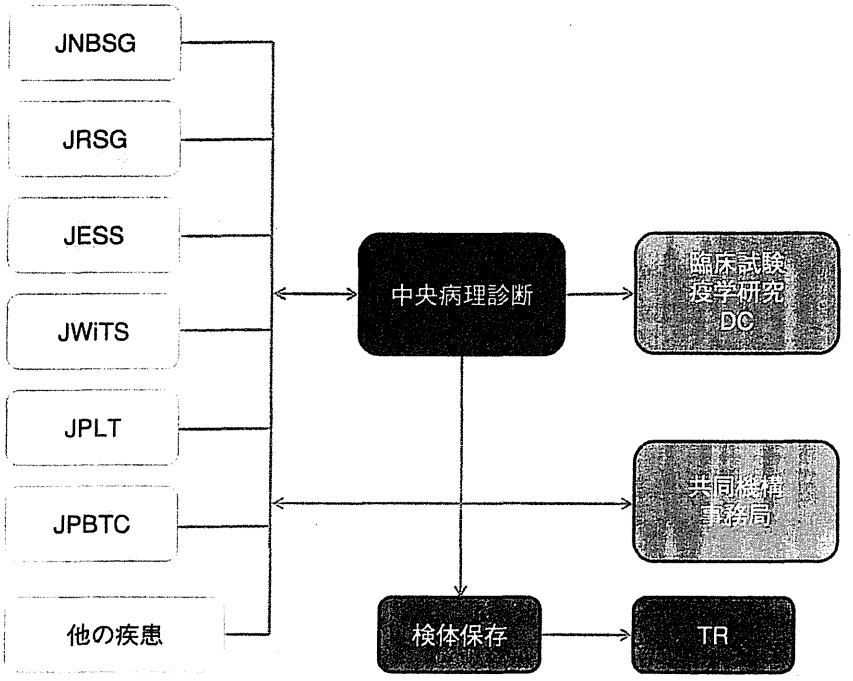

図 2 小児固形がん臨床試験共同機構観察研究の実施体制

定である。組織のスリム化も必要であり，現在，グループごとに開催されている運営委員会などもかなりの経費を要していることから，将来は共同機構の委員会として一本化し，そのうえで理事会や会員総会なども整備するなどの組織改編も必要と思われる。

おわりに

現在，厚生労働省では5年に一度のがん対策基本計画の見直しが行われている。今後の5年間は小児がん対策が重点項目となることが決定しており，ボールは国からわれわれに投げられることになる。今後，重点項目となることで国民の小児がん対策に対する期待はますます大きなものになることが予想され，国を代表する組織として小児白血病リンパ腫グループとともに目に見える成果をだし，かつ小児がん患者に貢献していく覚悟が必要である。また，一方では米国 COG のような組織を構築する絶好の機会でもある。しかし，共同機構の活動はまだ緒についたばかりであり，多くの課題がある。これらの課題に迅速に対応していくためには，小児がん診療医各位の協力と提言をお願いしたい。

* * *

小児がんは どのように治療されるのですか？

小児がんは成人がんと異なって，通常かなり大きくなってから発見されます．多くの場合，最初に手術で腫瘍を全部摘出しようとすると，腫瘍に巻き込まれている周りの重要な臓器，たとえば，腎臓，膀胱，大きな血管なども摘出しなくてはなりません．また，手足や首，顔，副鼻腔などに腫瘍がある場合は，手術により，手足の機能が失われたり，美容上，大きな問題が出たりします．このような場合は，最初は診断を目的とした手術（これを生検といいます）で腫瘍の一部だけを切り取って病理検査で診断を確定します．小児がんはほとんどの場合，抗がん剤がよく効きますので，その後抗がん剤治療（化学療法といいます）を数ヵ月行って腫瘍を小さくします．小さくなった腫瘍を手術で取り除いた後に，再び化学療法を行います．この間，必要に応じて放射線治療も実施します．小児がんは初期であっても全身に腫瘍細胞が散らばっているので，これらの細胞を殺すためにも化学療法は重要です．これらの治療をすべて完了するのに，半年から1年かかります．

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解　説

小児がんの年間発生数は白血病を含めて全国で約2,000人であり，全体の70～80％は治癒が期待できる．小児がんの治療は成人がんと異なる部分も多く，また，希少疾患であり（脳腫瘍，神経芽腫を除くと各疾患の年間発生数は100例以下），**一般的な病院では診療経験が限られるため，経験豊富な専門施設で診断も含めた治療が行われるべきである．また，小児がんのなかでもさらに特殊な知識や技能が要求されるような疾患，たとえば脳腫瘍，骨軟部肉腫，網膜芽細胞腫などは小児専門病院や大学病院でも診療実績が乏しい場合があるのでなおさらである．**また，小児がんの治療が患児の成長発達を妨げたり，疾患，治療の後遺症により患児が困ったりすることがないよう，臨床心理士，ホスピタルプレイスペシャリスト（チャイルドライフスペシャリスト）などの専門的技量を有する病棟保育士，教員（院内学級，養護学校）などのスタッフが治療中，治療終了後も関与する．一方，小児がん患児の20～30％は残念ながら最終的には死を避けることができない．そのため，緩和ケアが小児がん治療のなかに占める役割は大きく，終末期の患児とその家族の身体的，精神的苦痛を除去することが，極めて重要である．以下に，小児がん治療の各段階について述べる．

診　断

小児がんは希少であるがゆえ，小児腫瘍の経験が豊富な病理医でなければ正確な診断は困難である．そのため，最近では日本病理学会小児腫瘍分類委員会による中央診断と各施設病理医とのコンセス診断が行われる方向性にある．小児がんと診断された場合，進展度を決定するため，一般的な画像検査のほか，骨シンチグラフィー，骨髄検査，脳脊髄液検査などが必要に応じて行われる．

手　術

小児がんのほとんどはかなり増大してから発見されるため，周辺臓器などに進展している場合が多い．小児がんは化学療法の効果が大きいため，たとえば膀胱摘出や四肢切断などのように機能を大きく損なうような手術は行わないのが原則である．したがって，初診時は正常臓器の合併切除を行わずに一期的に腫瘍全摘が可能な場合以外は，病理診断を目的とした生検のみを行う．診断後，化学療法にて腫瘍を縮小せしめた後，腫瘍摘出を行うのが普通である．

化学療法

悪性腫瘍に対する抗がん剤治療(化学療法)の歴史は，1960年前後に小児がんから始まったほど，抗がん剤は小児がんに対して高い効果を示す．よく用いられる薬剤はアドリアマイシン，ビンクリスチン，シクロホスファミド，イホスファミド，アクチノマイシン，シスプラチン，カルボプラチン，エトポシドであり，ほとんどの小児がんはこれらのうちの3～6種類程度を組み合わせた多剤併用療法で治療される．化学療法は6ヵ月～1年間繰り返し行われる．化学療法は原発腫瘍や転移腫瘍を縮小，あるいは消失させるために行われるが，小児がんは全身への転移が早く，画像検査で同定されなくても診断時にはすでに微小な転移が全身に生じていることが多い．そのため，たとえ初診時に腫瘍が一期的に全摘された場合でも，これらの微小転移を消失させるために化学療法は必須である．小児では脳腫瘍でも化学療法が有効であり，とくに，髄芽腫，胚細胞腫瘍，全摘できない星細胞系腫瘍などでは化学療法の併用は必須である．小児がん領域における化学療法は多くの成人がんのような補助的なものではなく，がん治療の根幹をなすものでその成否が治療成績にかかわる．そのため，成人がんより化学療法は強力であり，副作用も強く出現する．主な副作用として，汎血球減少に伴う出血，敗血症のほか，腎障害，粘膜障害など致死的なものが多くあり，その出現頻度も高い．また，シクロホスファミドなどのアルキル化剤は投与量に応じて不妊や二次性腫瘍を引き起こす．エトポシドも使用後2～3年間は薬剤性白血病の危険性が高まる．このように化学療法の実施に当たっては，治療後時間を経て生じてくる障害(晩期合併症)も考慮に入れなければならない．したがって，化学療法は小児がん医療に精通した小児化学療法医によって行われる．

放射線治療

脳腫瘍を始めとして，小児がんでは手術，化学療法のほかに放射線治療が必要となることが多い．その適応は手術で腫瘍が完全に切除できない場合で，通常，腫瘍が最初に存在した部位に対して放射線治療が行われる．その毒性を軽減するため，1日当たり1.8～2Gy程度の線量で合計36～56Gyを20～30日間かけて照射する．放射線治療は化学療法と異なり，発達途上の小児では程度の差はあれ，晩期合併症の発生は必至である．たとえば，照射された骨や筋肉は発達障害を生じ，関節であれば，後年機能不全を生じる．また，顔面骨であれば後年変形を生じる．甲状腺や下垂体では線量が多ければホルモンの分泌障害が生じる．また，照射部位に新たながんを生じる可能性が高まる．このような晩期合併症をできる限り回避するため，必要部位以外にできるだけ照射しないような技術，すなわち3次元治療計画に基づいた強度変調照射や陽子線治療が欧米では主流になりつつあるが，わが国では実施可能な施設は少ないのが現状である．

緩和ケア

よくいわれることであるが，緩和ケアは診断時より始まる．臨床心理士などのスタッフによる心理的介入などの精神面でのサポートのほか，術後

疼痛や抗がん剤の副作用の口内炎の疼痛など，あらゆる痛みに対する疼痛管理を含めた緩和ケアが行われる．また，冒頭に述べたように終末期ケア，すなわち疼痛，呼吸困難などの不快な症状の軽減および除去，家族も対象とした心理的サポート，在宅療養などはきわめて重要である．

コ・メディカルによる介入

子どもは治療中も成長を続けており，治療中ともいえどもそれを促すための介入を行う．具体的には病棟保育士による遊びの提供，本の読み聴かせ，院内学級教員による教育などである．とくに学童期では，入院中の勉学の遅れが復学後の不登校につながるなど，小児がんが治癒した後の人生に大きな影響を与える．また，治療中の恐怖心や寂しさなどが心的外傷後ストレス障害などの原因となるため，チャイルドライフスペシャリスト(ホスピタルプレイスペシャリスト)などの専門的病棟保育士や臨床心理士による介入を行う．具体的には痛みを伴う処置や放射線治療や検査前のわかりやすい説明(プレパレーション)と実施中の付き添い(デストラクション)などを実施する．

おわりに

このように，小児がん治療は各診療科および種々の職種のコ・メディカルによる，集学的治療が行われる．**成長中という小児の特性と治癒後の長い人生に対する影響を考慮した治療と治療終了後の長期間のフォローアップというのが小児がん治療の最大の特徴である．**

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Clinical Cancer Research

Cancer Therapy: Clinical

Phase I Trial of a Glypican-3–Derived Peptide Vaccine for Advanced Hepatocellular Carcinoma: Immunologic Evidence and Potential for Improving Overall Survival

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Abstract

Purpose: The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target of anticancer immunotherapy against hepatocellular carcinoma (HCC). In this nonrandomized, open-label, phase I clinical trial, we analyzed the safety and efficacy of GPC3 peptide vaccination in patients with advanced HCC.

Experimental Design: Thirty-three patients with advanced HCC underwent GPC3 peptide vaccination (intradermal injections on days 1, 15, and 29 with dose escalation). The primary endpoint was the safety of GPC3 peptide vaccination. The secondary endpoints were immune response, as measured by IFN-γ ELISPOT assay, and the clinical outcomes tumor response, time to tumor progression, and overall survival (OS).

Results: GPC3 vaccination was well-tolerated. One patient showed a partial response, and 19 patients showed stable disease 2 months after initiation of treatment. Four of the 19 patients with stable disease had tumor necrosis or regression that did not meet the criteria for a partial response. Levels of the tumor markers α-fetoprotein and/or des-γ-carboxy prothrombin temporarily decreased in nine patients. The GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 patients. Furthermore, GPC3-specific CTL frequency after vaccination correlated with OS. OS was significantly longer in patients with high GPC3-specific CTL frequencies (N = 15) than in those with low frequencies (N = 18; $P = 0.033$).

Conclusions: GPC3-derived peptide vaccination was well-tolerated, and measurable immune responses and antitumor efficacy were noted. This is the first study to show that peptide-specific CTL frequency can be a predictive marker of OS in patients with HCC receiving peptide vaccination. *Clin Cancer Res; 18(13); 3686–96. ©2012 AACR.*

Introduction

While primary liver cancer, which predominantly consists of hepatocellular carcinoma (HCC), is the sixth most common cancer worldwide, it has a very poor prognosis, which makes it the third leading cause of cancer mortality (1). One of the major reasons for the poor prognosis of HCC is the limited availability of treatment options for advanced disease. The molecular-targeted agent sorafenib was recently proven to prolong overall survival (OS) in patients with advanced HCC and has become the standard drug for first-line systemic treatment (2, 3). However, according to Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is quite low, and the incidence of adverse drug reactions is high, especially in elderly patients (4). Moreover, no second-line treatment has been established for patients when sorafenib treatment has failed. Therefore, new treatment modalities are urgently required to prolong survival in patients with advanced HCC while minimizing the risk of adverse reactions.

Immunotherapy is a potentially attractive option for HCC. Many tumor antigens identified in HCC are potential antigens for peptide vaccines (5, 6). However, thus

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

A cancer vaccine that induces CTLs to tumor-associated antigens is a potentially attractive option for hepatocellular carcinoma (HCC). However, thus far, immunotherapy using tumor antigen–derived peptides has not showed a correlation between immunologic responses and antitumor efficacy in clinical trials in patients with advanced HCC. Glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC and correlates with poor prognosis.

In a phase I clinical study, we investigated the safety and antitumor effects of, and immunologic response to, a GPC3-derived peptide vaccine. Our results show that GPC3 peptide–specific CTLs appeared in peripheral blood and that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination.

This is the first study to show that peptide-specific CTL frequency was correlated with overall survival in patients with HCC receiving peptide vaccination. These observations suggest that GPC3-derived peptide vaccines could be a novel therapy for patients with HCC.

far, immunotherapy using tumor antigen–derived peptides has not showed adequate antitumor efficacy in clinical trials in patients with advanced HCC (7–9). The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC (72%–81%) and correlates with a poor prognosis (10–14). We identified HLA-A*24:02–restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) as peptides that can induce GPC3-reactive CTLs without inducing autoimmunity (15, 16). Moreover, by conducting a binding assay, we confirmed that HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can bind to HLA-A*02:06 and HLA-A*02:07. HLA-A24 is the most common HLA class I allele in the Japanese population, and 60% of Japanese individuals (95% of whom have an A*24:02 genotype), 20% of Caucasians, and 12% of Africans are positive for HLA-A24 (17, 18). HLA-A2 is also expressed in Japanese (40%) and other ethnic populations, with an estimated frequency of 50% in Caucasians (17, 19). In a preclinical study using a mouse model, we developed an optimal schedule for human clinical trials of a GPC3-derived peptide vaccine (20). On the basis of these results, we conducted a phase I clinical trial of this GPC3-derived peptide vaccine in patients with advanced HCC. We previously reported that several $GPC3_{144-152}$ peptide-specific CTL clones were established from peripheral blood mononuclear cells (PBMC) of patients vaccinated with HLA-A2–restricted $GPC3_{144-152}$ peptide in this trial (21). We recently completed this phase I clinical trial of the GPC3-derived peptide vaccine. We evaluated the vaccine's safety, tolerability, recommended phase II dose, and immunologic and clinical responses in this trial.

Materials and Methods

Patient eligibility

This phase I trial was approved by the Ethics Committee of the National Cancer Center and was carried out from February, 2007, to November, 2009. Patients with advanced or metastatic HCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of HCC on the basis of imaging modalities or histologic examinations; no expectation of response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–1; age between 20 and 80 years; no prior therapy within 4 weeks; life expectancy $\geq$3 months; HLA-A24- or HLA-A2–positive status, as determined using commercially available genomic DNA typing tests (Mitsubishi Chemical Medience); Child–Pugh liver function class A and B; and adequate organ function (white blood cell count $\geq$3,000/μL, hemoglobin $\geq$8.0 g/dL, platelets $\geq$50,000/μL, total bilirubin $\leq$3.0 mg/dL, aspartate aminotransferase $\leq$200 IU/L, alanine aminotransferase $\leq$200 IU/L, and serum creatinine $\leq$1.5 mg/dL). The following exclusion criteria were applied: massive ascites; known brain metastasis; pregnancy or lactation; known history of HIV infection; clinically serious infection; severe cardiac insufficiency; other active malignancy; history of organ allograft; immunodeficiency or history of splenectomy; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial, based on clinical judgment.

Study design and endpoints

This study was a nonrandomized, open-label, phase I clinical trial with dose escalation of the GPC3 peptides in patients with advanced HCC. HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL; American Peptide Company) was used in HLA-A24–positive patients and HLA-A*02:01–restricted $GPC3_{144-152}$ peptide (FVGEFFTDV; American Peptide Company) in HLA-A2–positive patients. Peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant (IFA; Montanide ISA-51VG, SEPPIC), by intradermal injection on days 1, 15, and 29. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines. Administration of 5 incremental doses of peptide (0.3, 1.0, 3.0, 10, and 30 mg/body) was planned. We planned administer each dose to 6 patients, including at least each 2 patients given HLA-A2 or A24-restricted peptide. The primary endpoint was the safety of peptide vaccination. The secondary endpoints were immunologic responses, clinical outcomes, and determination of the optimal dose of peptide for further clinical trials. 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. The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000001395).

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Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
0.3	1	75/M	II	III	0	B	C	TAE, PEI, RFA, S-1	PD	2	9	2402	1	8	+	1+	1+
	2	77/M	IV	IVB	0	A	C	PEI, Proton, TAE, TAI	SD	3	11	2402	0	5	+	1+	1+
	3	67/M	IV	IVB	0	A	–	Ope	SD	3	8	0206/0207	22	20	–	2+	1+
	4	51/M	IIIA	IVA	0	B	B	Ope, TAE, TAI	PD	1	2	0201	0	7	+	NA	NA
	5	62/M	IIIA	III	0	A	–	TAI	PD	2	5	0201	0	9	+	1+	1+
	6	69/M	IV	IVB	0	A	–		PD	0	1	0201	10	9	–	NA	NA
	7	59/M	IIIA	IVA	0	A	B	Ope, TAE, TAI	PD	2	3	2402	0	3	+	1+	1+
	8	55/M	IIIA	III	0	A	C	MCT, PEI, TAE, RT, Sor, S-1	SD	3	17	0201	1	5	+	1+	1+
1.0	9	68/F	IIIC	IVA	0	A	C	PEI, TAE, RFA	SD	4	13	0201	8	8	–	NA	NA
	10	72/M	IIIA	IVA	0	B	C	Ope, MCT, RFA, PEI, Sor, TAE, RT, S-1	SD	4	9	0201	8	51	+	1+	1+
	11	60/M	IIIC	IVA	0	A	C	TAE, RFA	SD	4	9	2402	0	11	+	–	1+
	12	62/M	II	III	0	A	–	RFA, PEI, TAE	PD	2	5	0201/0206	0	12	+	–	1+
	13	44/M	IV	IVB	0	A	B	TAE, RFA, PEI, RT	PD	2	24	2402	6	73	+	1+	2+
	14	42/F	IV	IVB	0	A	–		SD	4	14	2402	1	132	+	2+	1+
3.0	15	67/F	IV	IVB	0	A	–	Ope, PEI, TAE, Proton	SD	5	9	0201	0	23	+	1+	1+
	16	58/M	IIIA	III	0	A	–	Ope, TAE, S-1, TAE	SD	5	7	0201	0	101	+	1+	1+
	17	75/M	IIIC	IVA	0	A	C	RFA, TAE	PD	2	7	2402	0	69	+	–	1+
	18	70/M	IV	IVB	1	A	C	Ope, RT	SD	4	14	2402	0	72	+	1+	1+
	19	76/M	IIIA	III	0	B	C	Ope, TAE, TAI	SD	2	3	2402	31	68	+	1+	1+
	20	73/M	II	II	1	A	–	Ope, TAE	SD	8	>34	2402	0	124	+	1+	1+
10	21	52/M	IV	IVB	0	A	B	Ope, TAE, S-1	SD	4	8	0201	1	100	+	2+	1+
	22	71/M	IIIC	IVA	0	A	–	Ope	SD	4	>32	2402	0	171	+	–	1+
	23	70/M	IV	IVB	0	A	B	Ope, TAI, TAE, PEI	PD	2	6	0201	0	5	+	1+	–
	27	56/M	IV	IVB	0	A	C	TAE, UFT	SD	6	>23	2402	64	69	+	NA	NA
	28	57/M	IIIA	IVA	1	B	C	TAE, RFA, TAI	PD	1	1	2402	0	4	+	NA	NA
	29	68/M	IIIA	IVA	0	A	C	Ope, TAE, TAI	PD	2	4	0201	1	125	+	1+	2+
	33	76/M	IV	IVB	0	A	C	Ope, TAE, MCT, RFA, GEM	SD	4	>16	2402	0	5	+	2+	1+
30	24	75/F	IV	IVB	1	A	C	Ope, RFA, RT	PR	5	12	0207	11	196	+	1+	1+
	25	52/M	IV	IVB	0	A	B	Ope, RFA, TAE, RT, UFT	PD	2	12	0206	2	151	+	2+	2+

(Continued on the following page)

Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response (Cont'd)

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
	26	75/F	II	II	0	B	C	MCT, RFA, TAE, TAI	SD	2	8	2402	0	16	+	NA	NA
	30	69/M	IV	IVB	1	A	–	Ope, TAI, UFT, GEM+CDDP, RT	SD	4	6	2402	2	34	+	1+	–
	31	53/M	IV	IVB	0	B	B	TAE, RFA	SD	4	14	2402	0	7	+	NA	NA
	32	67/M	IV	IVB	0	A	B	Ope, Sor, TAE	PD	2	>17	0201	0	441	+	–	–

Abbreviation: PD, progressive disease; PFS, progression-free survival; PS, performance status.

[a]Stage: staging was carried out according to the TNM classification for HCC (Union for International Cancer Control, UICC) and the Japanese integrated staging system (Liver Cancer Study Group of Japan, LCSGJ).

[b]Hepatic virus infection B. HBsAg was examined by radioimmunoassay. C: HCV was detected by RT-PCR.

[c]Prior therapy: Ope, surgery; TAE, transcatheter arterial embolization; PEI, percutaneous ethanol injection therapy; RFA, radiofrequency ablation; S-1, tegafur, gimeracil, oteracil potassium; proton, proton beam therapy; TAI, transcatheter arterial injection; RT, radiotherapy; Sor, sorafenib; MCT, microwave coagulation therapy; UFT, tegafur plus uracil; GEM, gemcitabine; CDDP, *cis*-diamminedichloroplatinum.

[d]Tumor responses were evaluated according to RECIST guidelines and modified RECIST (mRECIST) assessment. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients.

[e]Number of GPC3-specific CTL spots. The number of GPC3 peptide–specific CTL spots (postvaccination) was the maximum number of spots in an *ex vivo* IFN-γ ELISPOT assay for GPC3 peptide, carried out after vaccination and using 5×10^5 PBMCs.

[f]Expression of GPC3 and HLA class I was determined by immunohistochemistry. Degree of staining of tumor cells for GPC3: –, no reactivity; 1+, weak reactivity; 2+, strong reactivity; NA, not analyzed. Degree of staining of tumor cells for HLA class I: –, no membranous reactivity; 1+, weak membranous reactivity; 2+, strong membranous reactivity; NA, not analyzed.

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Evaluation of toxicity and clinical response

Patients were evaluated for signs of toxicity during and after vaccination. Adverse events were graded according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Hematologic examinations were conducted before each vaccination. The tumor size was evaluated by computed tomography (CT) or MRI before vaccination, and then 1 month after the third vaccination. Tumor responses were evaluated according to the RECIST guidelines and the modified RECIST (mRECIST) assessment (22).

Measurement of immunologic response

Ex vivo *IFN-γ enzyme-linked immunospot assay.* An *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously (21). Briefly, peripheral blood (30 mL) was obtained from each patient before the first vaccination and 2 weeks after each vaccination and centrifuged with a Ficoll-Paque gradient. PBMCs were frozen before immunologic analysis. All PBMCs obtained from an individual patient were incubated in the same plate and analyzed by *ex vivo* IFN-γ ELISPOT assay at the same time. Noncultured PBMCs (5×10^5 per well) were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 hours at 37°C in 5% CO_2. The GPC3 antigen was the HLA-A2–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide or HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL). PBMCs plus HLA-A2–restricted HIV_{19-27} (TLNAWVKVV) peptide (ProImmune) or HLA-A*24:02–restricted $HIV_{583-591}$ (RYLKDQQLL; ProImmune) were used as negative controls. The assays were conducted in duplicate.

Dextramer staining and flow cytometric analysis. The PBMCs were stained with HLA-A*02:01 Dextramer-RPE [$GPC3_{144-152}$ (FVGEFFTDV), HIV_{19-27} (TLNAWVKVV); Immudex] and HLA-A*24:02 Dextramer-RPE [$GPC3_{298-306}$ (EYILSLEEL), $HIV_{583-591}$ (RYLKDQQLL); Immudex] for 10 minutes at room temperature and with anti-CD8-FITC (ProImmune) for 20 minutes at 4°C. Flow cytometry was carried out using a FACSAria cell sorter (BD Biosciences), as described previously (21).

Immunohistochemical analysis. Biopsy specimens were taken from some of the vaccinated patients, each of whom provided informed consent. Specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), HLA class I (clone EMR8/5; dilution 1:2,500; Hokudo), according to the manufacturers' directions.

GPC3 double-determinant (sandwich) ELISA. Double-determinant (sandwich) ELISA of GPC3 was carried out as described previously (10). The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12; BioMosaics Inc.), and anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (#211-GP/CF; R&D Systems).

Statistical analysis

OS rates were analyzed by the Kaplan–Meier method. Prognostic factors were evaluated using the log-rank test and Cox proportional hazard models. All statistical analyses were conducted using the PASW Statistics software, version 18.0 (SPSS Inc.). Statistical significance was defined by a value of P less than 0.05.

Results

Patient characteristics

Thirty-three patients were enrolled in this study (Table 1). None of the patients dropped out because of adverse events caused by peptide vaccination. Two patients (cases 4 and 6) discontinued the regimen after the second vaccination because of liver function impairment resulting from tumor progression. One patient (case 28) could not undergo a CT scan after the third vaccination because of tumor progression. These patients were judged to have disease progression, but were not removed from the analyses at the advice of the effect and safety evaluation committee, including the external members. All patients received adequate follow-up to monitor toxicity. The median follow-up period was 9.0 months (range, 1.1–34.1 months). Of the 33 patients, 28 were male. Their average age was 64.3 years (range, 42–77 years). Five patients had a performance status (PS) of 1; all others had a PS of 0. Staging was conducted according to the tumor-node-metastasis (TNM) classification for HCC (Union for International Cancer Control). Sixteen patients were diagnosed with stage IV disease. Seven patients had Child–Pugh class B disease, and all others Child–Pugh class A disease. Twenty-three patients (70%) had a hepatic virus infection. All but 2 of the 33 patients had undergone conventional chemotherapy, surgery, and transcatheter arterial embolization before receiving GPC3 peptide vaccine therapy. At the time of the trial's initiation, sorafenib had not been approved by the drug administration in Japan. Only a few patients had received sorafenib as prior therapy in this phase I trial. One patient treated with gemcitabine had had stable disease for 5 months immediately before vaccination (case 33). The gemcitabine therapy was discontinued because of nausea and lightheadedness. Other patients had undergone prior therapy, but all of them showed progression of the disease before enrollment in this study.

We evaluated the expression of GPC3 and HLA class I in the primary tumors that could be obtained (Supplementary Fig. S1). GPC3 expression was detected in 21 of 26 patients (81%), consistent with previous reports (10–14). Cell membrane expression of HLA class I was evident in 23 of 26 patients (88%; Table 1).

GPC3 peptide vaccine was well-tolerated

The adverse events observed in this trial are listed in Table 2. Dose-limiting toxicity and dose-specific adverse events were not seen. Grade III hematologic adverse events (impaired liver function) were observed in 4 patients (cases 4, 6, 7, and 23). These 4 patients had progressively massive

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Table 2. The incidence of adverse event

Adverse event	Total (%)	Grade I (%)	Grade II (%)	Grade III (%)
Any event	33 (100)	9 (27.3)	20 (60.6)	4 (12.1)
Any immune-related event	33 (100)	27 (81.8)	6 (18.2)	0
Drug fever	8 (24.2)	4 (12.1)	4 (12.1)	0
Rash or flushing	27 (81.8)	24 (72.7)	3 (9.1)	0
Injection site reaction	33 (100)	33 (100)	0	0
Pruritus	6 (18.2)	6 (18.2)	0	0
Blood	15 (45.4)	6 (18.2)	9 (27.3)	0
Leukopenia	6 (18.2)	2 (6.1)	4 (12.1)	0
Neutropenia	8 (24.2)	5 (15.2)	3 (9.1)	0
Anemia	5 (15.2)	2 (6.1)	3 (9.1)	0
Thrombopenia	3 (9.1)	1 (3.0)	2 (6.1)	0
Increase in PT-INR	2 (6.1)	2 (6.1)	0	0
Hepatic	23 (69.7)	10 (30.3)	9 (27.3)	4 (12.1)
Hyperbilirubinemia	9 (27.3)	3 (9.1)	4 (12.1)	2 (6.1)
Increase in aspartate aminotransferase	14 (42.4)	4 (12.1)	6 (18.2)	4 (12.1)
Increase in alanine aminotransferase	12 (36.4)	10 (30.3)	1 (3.0)	1 (3.0)
Renal	9 (27.3)	6 (18.2)	3 (9.1)	0
Increase in creatinine	4 (12.1)	2 (6.1)	2 (6.1)	0
Proteinuria	6 (18.2)	4 (12.1)	2 (6.1)	0
Other laboratory				
Increase in alkaline phosphatase	9 (27.3)	4 (12.1)	4 (12.1)	1 (3.0)
Hypoalubuminemia	10 (30.3)	7 (21.2)	3 (9.1)	0
Hyponatremia	13 (39.4)	12 (36.4)	1 (3.0)	0
Hyperkalemia	4 (12.1)	4 (12.1)	0	0

Abbreviation: PT-INR, prothrombin time-international normalized ratio.

liver tumors. The effect and safety evaluation committee, including the external members, judged that these events were not related to the treatment, but rather to disease progression. All patients experienced grades I or II local skin reactions at the injection site. Transient immune-related events, including drug fever, rash, and flushing, were observed in most patients. Crotamiton, a scabicidal and antipruritic agent, was prescribed to the 5 patients who had mild itching, but no antipyretic analgesics were prescribed. These results suggest that GPC3 peptide vaccine therapy was well-tolerated.

GPC3 peptide vaccination could induce peptide-specific CTLs in most patients

To determine whether the GPC3 peptide vaccine could induce a specific immune response, PBMCs, obtained from all patients before and after vaccination, were examined by *ex vivo* IFN-γ ELISPOT assay. After the second vaccination, the number of GPC3 peptide–specific CTLs in 5×10^5 PBMCs was increased from 0 to 441 in case 32 (Fig. 1A). As shown in Table 1, we found that the GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 of the 33 patients (91%). GPC3-specific CTL frequency increased in a peptide dose-dependent manner (Fig. 1B). Generally, CTLs for some tumor antigens cannot be directly detected *ex vivo*; they can only be detected after expansion by repeated *in vitro* stimulation with the antigenic peptide on appropriate antigen-presenting cells. This finding can be attributed to the sensitivity of the assay and the low frequency of tumor antigen–specific CTLs (23). Surprisingly, GPC3-specific CTLs were directly detected *ex vivo* without *in vitro* peptide stimulation in almost all patients after GPC3 peptide vaccination.

We also analyzed the GPC3-specific CTL frequency by flow cytometry using the GPC3 peptide, Dextramer. The GPC3-specific CTL frequency is indicated as the percentage of both Dextramer-positive and CD8-positice cells before and after vaccination, as shown in Fig. 1C. After the second vaccination, the frequency of GPC3-specific CTLs increased from 0% to 0.12% in case 32.

In many patients who were vaccinated only 3 times, the GPC3-specific CTL frequency decreased within 2 months after the third vaccination. We could vaccinate 4 or more times in 12 cases. In 9 of these, the GPC3-specific CTL frequency increased after the fourth vaccination (data not shown).

CTLs infiltrated the tumor after GPC3 peptide vaccination

Tumor biopsy was carried out (with informed consent) in 7 patients to evaluate the therapeutic effect after vaccination. We evaluated infiltration of CD8-positive T cells by

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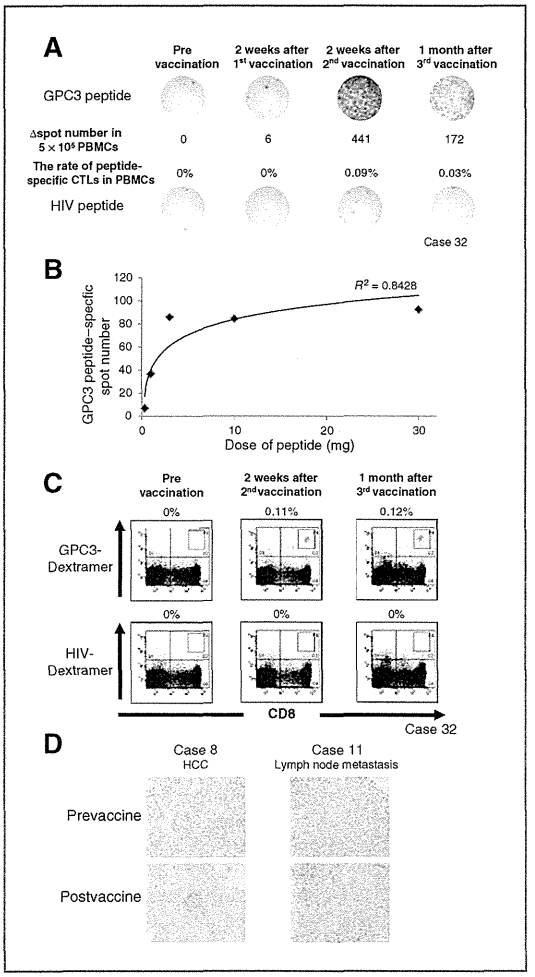

Figure 1. Immunologic monitoring of GPC3 peptide–specific T-cell responses. A, *ex vivo* IFN-γ ELISPOT assay for GPC3 in 5×10^5 PBMCs was carried out before and after vaccination in case 32. The Δspot number indicates the number of GPC3 peptide–specific CTLs. The number of IFN-γ–positive spots increased from 0 to 441 in the wells preincubated with GPC3 peptide. B, median spot number in *ex vivo* IFN-γ ELISPOT assay for GPC3 for each peptide dosage. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. C, *ex vivo* GPC3 Dextramer staining before and after vaccination in case 32. GPC3 peptide–specific CTL frequency is indicated as the percentage of Dextramer-positive CTLs among PBMCs. The frequency of GPC3 peptide–specific CTLs increased from 0% to 0.12% in case 32. D, immunohistochemical staining showing CD8-positive lymphocytes infiltrating tumors before and after vaccination. In cases 8 and 11, CD8-positive T cells (brown) did not infiltrate the tumors before vaccination; in contrast, many CD8-positive T cells infiltrated the tumor after vaccination. Magnification, ×200.

immunohistochemical staining. In case 8, liver biopsy was carried out before and after vaccination. In case 11, neck lymph node metastasis was resected after vaccination. The specimen was compared with an abdominal lymph node metastasis sample obtained by a diagnostic biopsy that this patient underwent before vaccination. While CD8-positive T cells did not infiltrate the tumor before vaccination, marked infiltration of CD8-positive T cells into the tumor was observed after vaccination in both cases (Fig. 1D). In 5 of 7 cases, infiltration of CD8-positive T cells into the tumor was increased after vaccination.

Clinical responses

Patient characteristics and clinical responses in relation to GPC3-specific CTLs are shown in Table 1. Among the 33 patients, one (case 24) was judged to have a partial response (PR) and 19 patients stable disease (SD) for 2 months, according to RECIST. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients. The disease control rate (PR + SD) was 60.6% after 2 months. The median time to tumor progression (TTP) was 3.4 months [95% confidence interval (CI), 2.1–4.6]. The median OS was 9.0 months (95% CI, 8.0–10.0).

In case 24, supraclavicular lymph node metastases markedly regressed, 2 liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (Fig. 2A and B). We carried out a biopsy of the remaining liver tumor and the thoracic bone metastasis after obtaining informed consent. Immunohistochemical staining showed expression of GPC3 and HLA class I on cells in the remaining liver tumor (Fig. 2C). Surprisingly, we detected massive infiltration of CD8-positive T cells into the remaining liver tumor by immunohistochemical staining. No viable tumor cells were found in the biopsy specimens of the thoracic bone metastasis.

Four other patients (cases 1, 15, 16, and 17) had tumor necrosis or partial tumor reduction that did not meet the PR criteria.

Serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are useful tumor markers of HCC (24). The levels of AFP or DCP decreased temporarily at least once in 9 of the 33 patients during the 2-month period (Supplementary Table S1). In 7 of these 9 patients, the levels of DCP fell to less than 30% of baseline values. In 15 of 32 patients, GPC3 protein was detectable in serum before vaccination. The serum levels of GPC3 temporarily decreased at least once in 12 of these 15 patients (data not shown).

These results suggest that there is not the duration of the responses in regards to CTL induction and tumor responses in this phase I trial.

OS was correlated with GPC3-specific CTL frequency

We also examined prognostic factors (Table 3). Fifty GPC3 peptide–specific CTL spots were detected in an *ex vivo* IFN-γ ELISPOT assay conducted using 5×10^5 PBMCs, which means that the GPC3 peptide–specific CTL frequency in peripheral lymphocytes was is 1×10^{-4}%. We focused on these 50 spots to elucidate prognostic factors. Univariate analysis indicated that distant metastasis (−; $P = 0.032$), invasion of the inferior vena cava (IVC) or portal vein (PV;

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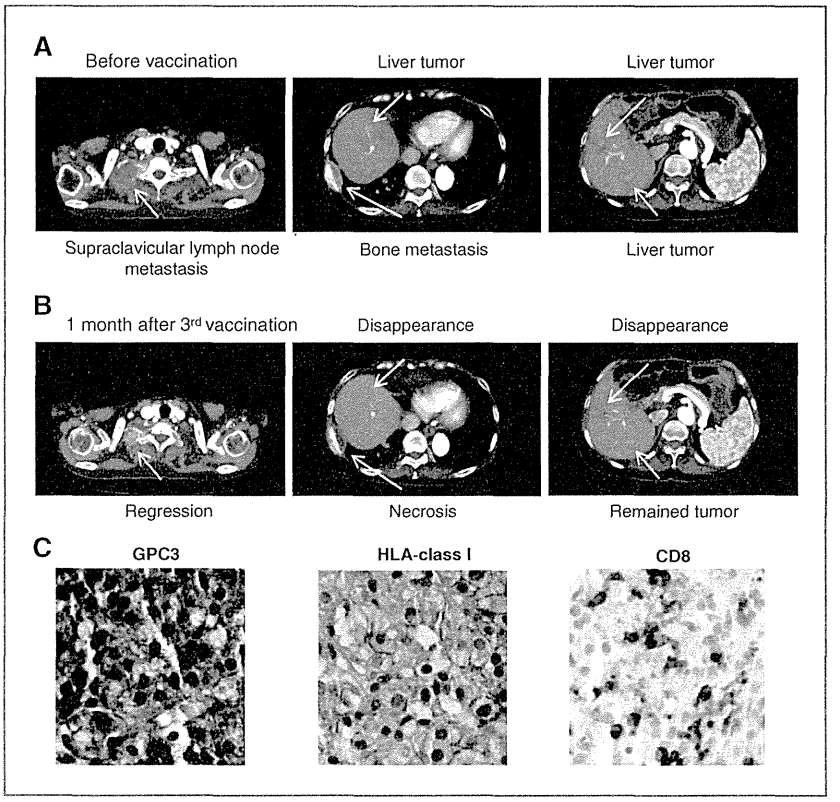

Figure 2. Response assessment in case 24. A, CT imaging, showing liver, pleura, and supraclavicular lymph node metastases before vaccination. B, CT imaging after vaccination was judged as an indicator of a PR. The supraclavicular lymph node metastasis and multiple liver tumors regressed markedly. The pleura metastasis was necrotic. C, we biopsied the remaining liver tumor after vaccination. Immunohistochemical staining showed expression of GPC3 and HLA class I on tumor cells. There was massive infiltration of CD8-positive T cells. Magnification, ×200.

$P = 0.040$), AFP ≥ 100 ng/mL ($P = 0.003$), tumor size ≥ 10 cm ($P = 0.003$), and GPC3-specific CTL frequency < 50 were prognostic factors for OS. Furthermore, AFP ≥ 100 ng/mL ($P = 0.004$; HR = 4.66; 95% CI, 1.61–13.19), tumor size ≥ 10 cm ($P = 0.003$; HR = 4.36; 95% CI, 1.58–12.05), and GPC3-specific CTL frequency < 50 ($P = 0.032$; HR = 2.71; 95% CI, 1.09–6.72) were prognostic factors for OS in a multivariate analysis. We showed that GPC3-specific CTL

Table 3. Prognostic factors of OS

	P univariate	*P* multivariate	HR (95% CI)
Sex (male/female)	0.991		
Age (≥65/<65)	0.608		
Performance status (0/1)	0.707		
Child–Pugh (A/B)	0.063		
Virus infection (+/−)	0.956		
Distant metastasis (+/−)	0.032	0.284	1.71 (0.64–4.54)
Invasion of IVC or PV (+/−)	0.040	0.706	1.21 (0.45–3.30)
AFP (≥100/<100 ng/mL)	0.003	0.004	4.66 (1.61–13.19)
Tumor size[a] (≥10/<10 cm)	0.003	0.005	4.36 (1.58–12.05)
GPC3-specific CTL[b] (≥50/<50)	0.033	0.032	2.71 (1.09–6.72)
HLA (A2/A24)	0.091		
Vaccine[c] (≥1/<1 mg)	0.053		

[a]Tumor size estimated by the RECIST.
[b]The GPC3 peptide–specific CTL frequency examined with *ex vivo* IFN-γ ELISPOT assay in 5×10^5 PBMCs.
[c]The dosage of one vaccine.

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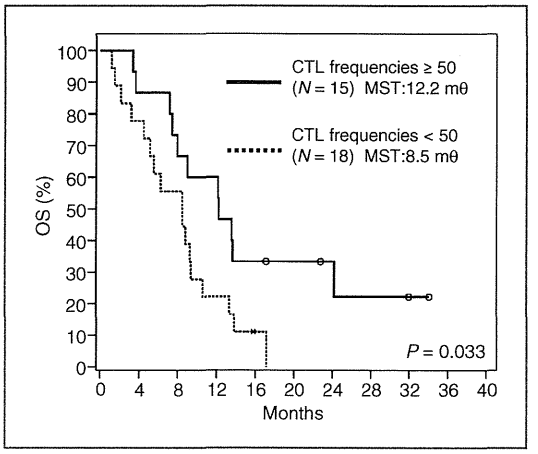

Figure 3. Kaplan–Meier curves for OS. Patients with GPC3-specfic CTL frequencies ≥50 had a longer survival than those with GPC3-specfic CTL frequencies <50 (P = 0.033). MST, median survival time.

frequency could be a predictive marker of the effects of GPC3 peptide vaccination. We compared patients with GPC3-specfic CTL frequencies ≥ 50 (N = 15) with those with GPC3-specific CTL frequencies < 50 (N = 18) and found that there was no significant difference in clinical background. We only found a significant difference (P = 0.004) for vaccine consumption (≥1.0 vs. <1.0 mg; Supplementary Table S2). Analysis of all 33 patients showed that the median OS was 12.2 months (95% CI, 6.5–18.0) in patients with GPC3-specfic CTL frequencies ≥50, compared with 8.5 months (95% CI, 3.7–13.1) in those with GPC3-specfic CTL frequencies <50 (P = 0.033; Fig. 3).

Discussion

We did not observe dose-limiting toxicity in this study. It was difficult to determine the maximum tolerated dose of peptide. A peptide dose of greater than 1.0 mg was required for adequate induction of GPC3-specific CTLs. However, it was complicated to inject more than 10 mg of peptide intradermally because injection mixtures contained both peptide and IFA, and doses of peptide vaccine >10 mg emulsified with IFA (consisting of 2 mL of fluid, including 1 mL of IFA), increased local skin reactions (induration, blushing) at the injection site (Supplementary Fig. S2). Therefore, a dose of peptide of 3.0 mg is recommended for future clinical trials.

We evaluated the expression of GPC3 in the primary tumors of 26 patients by immunohistochemistry. Among the 21 patients with low GPC3 expression (degree of staining − or 1+), one patient was judged to have a PR, and 3 patients have shown long-term survival. We do not suggest that only patients with high GPC3 expression (degree of staining 2+) should be enrolled in further clinical trials.

We studied immunologic responses using an *ex vivo* IFN-γ ELISPOT assay. The GPC3 peptide vaccine induced GPC3-specific CTL responses in 30 of the 33 patients. In contrast, clear immune responses were not observed in patients with HCC in another vaccination trial (9). Differences in tumor antigen may account for the differences in immune response between the 2 vaccination trials. Previous studies have shown that GPC3 is also overexpressed in other malignant tumors, including melanomas, Wilms' tumor, hepatoblastoma, ovarian clear cell carcinoma, and lung squamous cell carcinoma (12, 25–28). GPC3 might also be an effective target for immunotherapy against these tumors (29, 30).

In our study, none of the patients in the 0.1 mg dose group showed more than 50 GPC3 peptide–specific CTL spots. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. Previously, Salgaller and colleagues reported no dose dependency in the capacity of the gp100 peptide to enhance immunogenicity in humans (at doses 1.0–10 mg; ref. 31). In contrast, our data indicate dose dependency in CTL induction, consistent with a previous report using a mouse model (20).

Ten of the 25 patients who received a dose higher than 1.0 mg did not exhibit GPC3-specfic CTL frequencies ≥50. There was no significant difference in the clinical background of patients with GPC3-specific CTL frequencies ≥50 and those with <50. However, GPC3-specific CTL frequency tended to correlate with the serum level of AFP or summed intrahepatic tumor size (Supplementary Table S2). In this study, several patients with advanced HCC exhibited a poor immunologic response to GPC3 peptide vaccination. There are several possible explanations for this poor immunogenicity. HCC is frequently accompanied by cirrhosis, which creates an immunosuppressive environment. There is impairment of the function and maturation of dendritic cells, which has been shown to be related to an imbalance in the extracellular amino acid profile (32). In progressive HCC, the induction of CTL may be suppressed by regulatory T cells or immunosuppressive cytokines (33). It has been reported that GPC3-specific CTLs become exhausted in HCC, and that this exhausted state cannot be reversed by blocking the CTLA-4 and PD-1 inhibitory costimulation pathways (34). Further studies will be necessary to increase the clinical efficacy of immunotherapy for advanced HCC.

The primary endpoint of this study was assessment of the safety of vaccination, but we also showed that tumor antigen–specific CTLs had a crucial role in the immunotherapy against GPC3. GPC3-specific CTL frequency was correlated with OS in this study. Peptide-specific IgG and delayed-type hypersensitivity postvaccination have been reported as potential predictive makers of prolonged survival in patients with advanced cancer vaccinated with peptides (35, 36). However, correlations between immune responses and OS have not been reported in other immunotherapy trials for HCC (7–9, 37). We found that patients with GPC3-specfic CTL frequencies ≥ 50 had a longer survival than those with GPC3-specfic CTL frequencies <50. There was no significant difference in the clinical backgrounds of patients with GPC3-specific CTL frequencies ≥50 and those with <50.

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We clearly showed the presence of GPC3 peptide–specific CTLs in peripheral blood, and showed that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination. The evidence in this study serves as a proof-of-concept for immunotherapy using tumor antigen–specific CTLs. However, we did not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide–specific CTLs. We are currently initiating a pilot study of liver biopsies carried out before and after GPC3 peptide vaccination for advanced HCC to determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide–specific CTLs.

No complete responses were observed when GPC3 peptide vaccination was used as the sole therapy for advanced HCC. To-date, there has been no report of an adequate antitumor efficacy of immunotherapy in clinical trials involving patients with advanced HCC; however, immunotherapy, as an adjuvant after surgical resection, is expected (38). On the basis of this study, we have begun a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC and have also planned combinatorial approaches with chemotherapy.

In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe and indicated a plethora of immunologic responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Kuronuma, T. Takayama, K. Uesaka, J. Furuse, T. Nakatsura
Development of methodology: J. Furuse
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sawada, T. Yoshikawa, D. Nobuoka, H. Shirakawa, Y. Motomura, H. Ishii, K. Nakachi, M. Konishi, S. Takahashi, N. Gotohda, J. Furuse, T. Kinoshita, T. Nakatsura
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sawada, S. Mizuno, J. Furuse, T. Nakatsura
Writing, review, and/or revision of the manuscript: Y. Sawada, T. Yoshikawa, D. Nobuoka, T. Takayama, J. Furuse, T. Nakatsura
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Furuse
Study supervision: T. Nakagohri, T. Takayama, K. Yamao, J. Furuse
Monitoring and evaluation of data and safety of the study: K. Uesaka

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A glypican-3-derived peptide vaccine against hepatocellular carcinoma

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Keywords: clinical trial, cytotoxic T lymphocyte, glypican-3, hepatocellular carcinoma, peptide vaccine

The results of a Phase I clinical trial in which a glypican-3 (GPC3)-derived peptide was tested in advanced hepatocellular carcinoma patients point to a strong correlation between immunological and clinical responses. This commentary reviews our fundamental studies and clinical trials on the GPC3-derived peptide vaccine.

The induction of tumor-specific responses in the absence autoimmunity is the ideal goal of immunotherapy. Since the identification of tumor-associated antigens in hepatocellular carcinoma (HCC), immunotherapeutic approaches have been based on the generation of tumor-specific $CD8^+$ T cells that recognize peptides of 8–11 residues derived from intracellular proteins and presented in association with MHC Class I molecules.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans, which are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. We identified GPC3 as a carcinoembryonic antigen and suggested that it would consitute an ideal target for HCC immunotherapy, due to its specific overexpression in HCC (in 81% of paitents) and its correlation with poor prognosis.[1–4] Furthermore, we identified both HLA-A24*(A*2402)*-restricted and H-$2K^d$-restricted $GPC3_{298–306}$ (EYILSLEEL), as well as HLA-A2*(A*0201)*-restricted $GPC3_{144–152}$ (FVGEFFTDV), as peptides that can induce GPC3-reactive cytotoxic T lymphocytes (CTLs) but not autoimmunity.[2,5] HLA-A24 and A2 are the most common MHC Class I alleles in the Japanese population. By performing a binding assay, we confirmed that the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide can also bind to HLA-A*02:06 and HLA-A*02:07. We then conducted a preclinical study in mice to design an optimal schedule for a clinical trial with the GPC3-derived peptide vaccine (**Fig. 1**). This study showed that incomplete Freund's adjuvant (IFA) is indispensable for GPC3 peptide-based immunotherapy, and that the immunological effects of the peptide vaccine are dose-dependent.[6]

Based on these results, we conducted a Phase I clinical trial using this GPC3-derived peptide vaccine in patients with advanced HCC, which has recently been concluded.[7] In this study, 33 patients with advanced HCC received GPC3 peptide vaccination with dose-escalation. Peptides were emulsified with IFA and administered in liquid form by intradermal injection on days 1, 15 and 29. The $GPC3_{298–306}$ peptide was used in HLA-A24-positive patients and the $GPC3_{144–152}$ peptide in HLA-A2-positive patients.

In this trial, we collected evidence of immune responses, demonstrated antitumor effects, and demonstrated the safety of our GPC3-derived peptide vaccine. One patient manifested a partial response (PR) and 4 out of 19 patients with stable disease (SD) exhibit tumor necrosis or regression that did not meet the criteria for PRs. Two months after initiation of treatment, the disease control rate (PR+SD) was 60.6%. When we analyzed the frequency of GPC3-specific CTLs ex vivo by interferon γ (IFNγ) enzyme-linked immunospot (ELISPOT) assays, we could detect GPC3 peptide-specific CTLs in the peripheral blood of most patients. Alongside, we established several $GPC3_{144–152}$ peptide-specific CTL clones from peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial.[8] Tumor biopsies were performed in seven patients to evaluate the infiltration of $CD8^+$ T cells by immunohistochemistry. In five cases, we observed a marked intratumoral infiltration of $CD8^+$ T cells upon vaccination.

A correlation between immunological and clinical responses is nowadays a required as proof for the clinical efficacy of immunotherapy. The frequency of GPC3 peptide-specific CTLs in the peripheral blood correlated with overall survival in HCC patients who received the peptide vaccination. In multivariate analysis, the frequency of GPC3-peptide-specific CTLs constitute the only predictive factor for overall survival in this trial. Analysis of all 33 patients showed a median overall survival of 12.2 mo (95% CI, 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs, compared with 8.5 mo (95% CI, 3.7–13.1) in individuals with a low GPC3-specific CTL frequency ($p = 0.033$). These observations suggest that GPC3-derived peptide vaccines represent a novel immunotherapeutic strategy for patients with HCC, with a potential to improve overall survival.

We subsequently conducted a Phase II study of the GPC3-derived peptide vaccine

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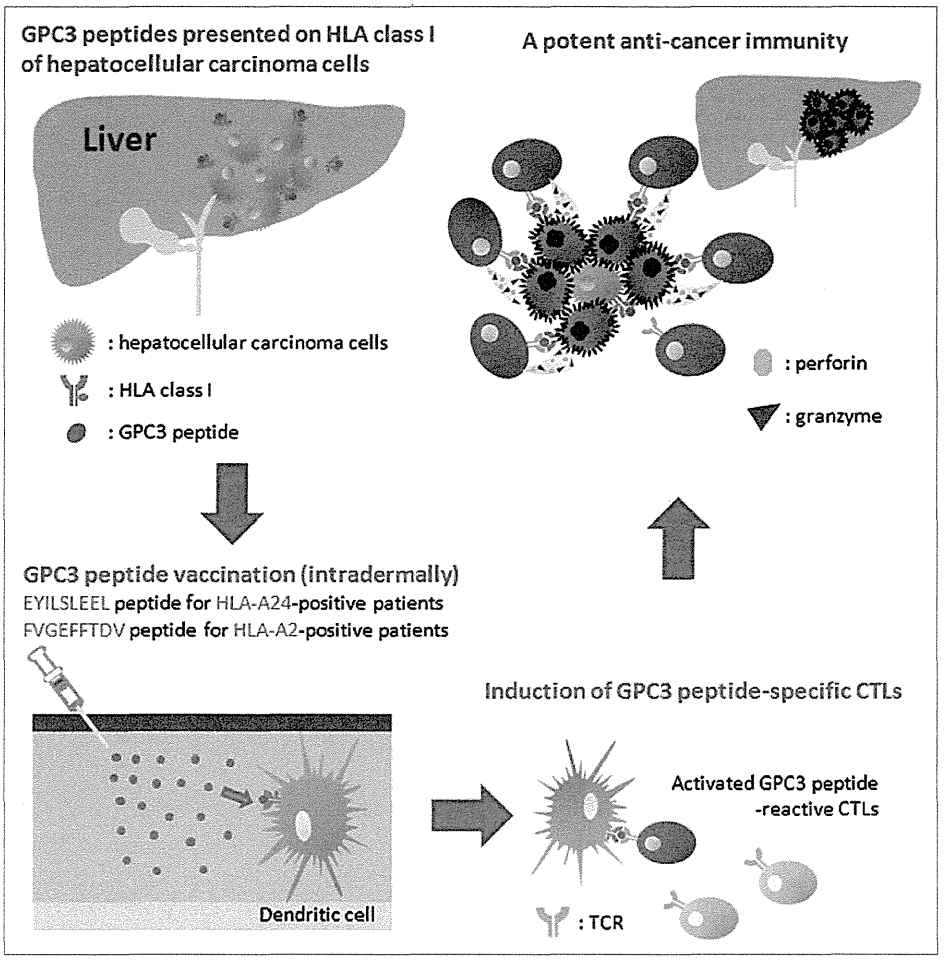

Figure 1. Mechanism of action of the GPC3-derived peptide vaccination. Most patients with hepatocellular carcinoma (HCC) exhibit an HLA-restricted glypican-3 (GPC3)-derived peptide presented in association with MHC Class I molecules. In clinical trials based on GPC3-derived peptide vaccines in HCC patients, the $GPC3_{298-306}$ (EYILSLEEL) peptide was used in HLA-A24-positive patients and the $GPC3_{144-152}$ (FVGEFFTDV) peptide in HLA-A2-positive patients. The peptides were administered with incomplete Freund's adjuvant by intradermal injection, leading to engulfment and cross-presentation by dendritic cells. Dendritic cells are capable of inducing GPC3 peptide-specific cytotocxic T lymphocytes (CTLs), which mediate anticancer immune responses.

as an adjuvant therapy for patients with HCC (UMIN-CTR: 000002614). Forty patients with HCC who had undergone surgery or radiofrequency ablation were enrolled in this Phase II, open-label, single-arm trial. Ten vaccinations were performed over 1 year after curative treatment. Primary endpoints were the 1- and 2-year recurrence rates, while secondary endpoints were immunological responses, as measured by IFNγ ELISPOT. The correlation between the time of recurrence and immunological responses is currently being analyzed.

In the Phase I trial, we did not confirm whether the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific. To address this issue, we are initiating a pilot study of liver biopsies performed before and after GPC3 peptide vaccination for advanced HCC (UMIN-CTR: 000005093).

GPC3 is overexpressed in several malignant tumors, including ovarian clear cell carcinoma (CCC), which is normally characterized by a poor prognosis due to low sensitivity to conventional chemotherapy. We confirmed that a $GPC3_{144-152}$ peptide-specific CTL clone can recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines using an IFNγ ELISPOT assay, and that is can kill ovarian CCC cell lines.[9] We are currently conducting a Phase II study with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

We expect that the results of these trials will provide a rationale for larger randomized clinical trials that determine the efficacy of GPC3-derived peptide vaccines. In addition, as the antitumor effect of the peptide vaccine alone is not dramatic in advanced cancer patients, we aim to develop combinational approaches[9] or strong antigen-specific immunotherapies, including adoptive cell transfer approaches following lymphodepletion.[10] Finally, clinical trials of the adoptive cell transfer of GPC3-specific CTLs in patients with HCC in Japan are planned. Well-designed clinical trials using innovative immunotherapeutic approaches will lead to the development of efficient new therapies for the treatment of GPC3-expressing tumors.

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Identification of an H2-K^b or H2-D^b restricted and glypican-3-derived cytotoxic T-lymphocyte epitope peptide

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Abstract. Glypican-3 (GPC3) is overexpressed in human hepatocellular carcinoma (HCC) but not expressed in normal tissues except for placenta and fetal liver and therefore is an ideal target for cancer immunotherapy. In this study, we identified an H2-K^b or H2-D^b restricted and murine GPC3 (mGPC3)-derived cytotoxic T-lymphocyte (CTL) epitope peptide in C57BL/6 (B6) mice, which can be used in the design of preclinical studies of various therapies with GPC3-target immunotherapy *in vivo*. First, 11 types of 9- to 10-mer peptides predicted to bind with H2-K^b or H2-D^b were selected from the mGPC3 amino acid sequence based on the binding score as calculated by the BIMAS software. We evaluated the peptide-binding affinity and confirmed that all peptides were able to bind to H2-K^b or H2-D^b by *in vitro* cellular binding assay. Subsequently, a mixed peptide vaccine and single peptide vaccine were given to B6 mice to evaluate immunogenic potential of the 11 selected peptides. Using the splenocytes from peptide-vaccinated mice, interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays showed that mGPC3-1$_{127\text{-}136}$ (AMFKNNYPSL) peptide was the most efficient for inducing CTLs among the 11 peptides. Next, we demonstrated that the mGPC3-1 peptide-specific CTL line could recognize mGPC3-expressing cancer cells, suggesting that mGPC3-1 peptide was an endogenously presented peptide. In conclusion, we identified mGPC3-1 as an H2-K^b or H2-D^b restricted, mGPC3-derived CTL epitope peptide.

Introduction

Liver cancer ranks fifth in frequency in the world and is the third most common cause of lethal cancer (1). Liver cancer consists of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), with HCC as the most common. Regarding HCC therapy, hepatectomy, percutaneous local therapy and transcatheter arterial embolization (TAE) are common, but the recurrence rate with conventional therapies for advanced HCC patients is still high (2). Therefore, developing a novel curative therapy or an effective adjuvant therapy for HCC is important.

Recently, immunotherapy, which consists of a peptide vaccine, protein vaccine, or DNA vaccine, has become a potentially promising option for HCC (3,4). Many tumor antigen-derived peptides recognized by cytotoxic T-lymphocyte (CTL) have been identified (5). However, to date, vaccine therapy using these peptides has not proven adequate antitumor efficacy in clinical trials for advanced HCC patients (6-8).

In HCC, glypican-3 (GPC3) is overexpressed and is not expressed in normal tissues except for the placenta and embryonic liver (9). Hence, GPC3 is a novel target molecule in HCC patients. GPC3 is a member of the heparan sulfate proteoglycan family and the glypican family regulates cell growth and division through Wnt signaling, Hedgehogs, fibroblast growth factors and bone morphogenetic proteins (10-12). We previously identified HLA-A*24:02-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A*02:01-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV) peptides and showed that both peptides can induce GPC3-specific CTLs without an autoimmune response (13,14). Clinical trials of a GPC3-derived peptide vaccine for HCC patients are currently in progress. The phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC showed safety as well as immunological evidence and potential for improving overall survival (15-17). The phase I clinical trial suggested that the GPC3-derived peptide vaccine could be an attractive approach for treatment of HCC, however, the effect of tumor reduction was limited. Therefore, further studies are needed to enhance the effect of GPC3-targeted immunotherapy and to establish a GPC3-specific CTL-inducible mouse model. We previously conducted a preclinical study of the GPC3-derived peptide vaccine using HLA-A2.1 transgenic mice (18). The treatment model experiment using HLA transgenic mice is limited.

Mice with the C57BL/6 (B6) background have been reported to spontaneously develop liver cancer (19,20). Recently, the NASH mouse model (named STAM mice C57BL/6N-NASH), which had a B6 background and spontaneously developed liver

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