り，それらを標的とした分子標的薬の開発が急速に進歩してきた。肝細胞癌においても従来の化学療法に代わって多くの分子標的薬が試みられてきている[6]。多くの分子標的薬のなかで，これまで肝細胞癌において表1の薬剤の臨床試験が行われている[6]。

これらのなかでsorafenibはわが国において日本人肝細胞癌患者での第Ⅰ相試験が行われた[7]。その結果，他癌腫や米国・ヨーロッパ人と同様の薬物動態および忍容性が確認され，推奨用量も400 mg，1日2回と同様の用法用量と決定された。同試験では症例数は少ないものの，奏効率4％，PFS中央値4.9ヵ月，OS中央値15.6ヵ月と欧米での第Ⅱ相試験と同等以上の有効性も確認された。その後，プラセボコントロールによるRCT SHARP (Sorafenib HCC Assessment Randomized Protocol) trialが実施され，OS中央値はsorafenib群10.7ヵ月，プラセボ群7.9ヵ月であり，ハザード比0.69 (95％CI：0.55-0.87，p＝0.0006)と両者間に明らかな統計学的有意差

表1　肝細胞癌において臨床試験の行われた主な分子標的薬

erlotinib：	epidermal growth factor receptor (EGFR) tyrosine kinase阻害薬 奏効率0～9％，PFS中央値3.1～3.2ヵ月，OS中央値6.3～13.0ヵ月
cetuximab：	EGFRに対する抗体薬 奏効率0％，PFS中央値1.4ヵ月，OS中央値9.6ヵ月
bevacizumab：	vascular endothelial growth factor(VEGF)を中和する抗体薬 単剤：奏効率8％，PFS中央値6.5ヵ月，OS中央値記載なし erlotinib併用：奏効率21％，PFS中央値9.0ヵ月，OS中央値19.0ヵ月
sorafenib：	RAFキナーゼとVEGFR-1-3，PDGFRなどマルチキナーゼ阻害薬 奏効率2％，PFS中央値4.2ヵ月，OS中央値9.2ヵ月
sunitinib：	VEGFR-2，PDGFR，c-Kitなどマルチキナーゼ阻害薬 奏効率4％，PFS中央値4.1ヵ月，OS中央値11.6ヵ月

（文献6より引用）

を認めた[8]。さらに無増悪期間(time to progression)中央値も sorafenib 群 5.5 ヵ月，プラセボ群 2.8 ヵ月，ハザード比 0.58 (95 % CI : 0.45-0.74，p = 0.000007)と明らかな統計学的有意差を認めた。sorafenib は十分な忍容性があり，進行肝細胞癌患者の生存期間を延長した初めての全身治療であり，今後標準的化学療法と位置づけられるものと考えられる。

胆道癌

1. 切除不能例に対する化学療法

化学療法の適応は切除不能の局所進行や遠隔転移を有する例，あるいは切除後の再発例に限られる。胆道癌診療ガイドラインでは化学療法は全身状態が良好な例に適応することが推奨されている[4]。全身状態の低下した例，すなわち PS 2，3 の場合や減黄不良例などでは化学療法の利益は少なく，適応は慎重に考慮すべきである。このような患者では，疼痛コントロール，閉塞性黄疸に対する胆管内ステントの留置など quality of life (QOL)の維持を目指した緩和治療を行う。

胆道癌の化学療法ではこれまで多くの第 II 相試験が行われてきている。最近の第 II 相試験をみると，GEM，CDDP あるいは L-OHP の白金製剤，capecitabine など新規フッ化ピリミジン剤が中心となっている。これらのなかで，GEM +白金製剤，GEM + capecitabine で奏効率 21 ～ 45 %，OS 中央値 4.6 ～ 15.4 ヵ月と良好な成績が報告されている[4]。

胆道癌では，胆嚢癌，胆管癌，乳頭部癌を含む多様性，減黄状況，PS，進行度など治療成績に関連する因子が多いため，単アームによる試験の治療成績の評価は難しい。したがって，RCT が必須であるが，これまで十分な患者数による第 III 相試験は行われていないし，統計学的に意義のある有効な治療法も報告されていない。英国で行われた GEM 単独と CDDP + GEM 併用の無作為化第 II 相試験では，PFS 中央値が GEM 群で 4 ヵ月，CDDP + GEM 群で 8.0 ヵ月と併用療法で有効性が期待され，その後大規模第 III 相試験に進んでいる[9]。

わが国では 2002 年以降，GEM や S-1 単独療法の治験が行われている。GEM では奏効率 17.5 %，OS 中央値 7.6 ヵ月と単剤としては良好な成績が得られ[10]，2006 年 6 月，保険適応の承認が得られた。引き続き，英国の比

較試験を参考にしたGEM単独とCDDP＋GEMのRCT第Ⅱ相試験が行われている。S-1では奏効率32.5％，OS中央値9.4ヵ月とさらに良好な成績が得られ[11]，2007年8月保険適応に承認された。胆道癌診療ガイドラインでは有効な抗癌剤としてGEMまたはS-1が挙げられている[4]。今後，GEM＋S-1併用療法なども含め，第Ⅲ相試験の実施により標準治療の確立が必要である。

2. 切除後補助化学療法

膵癌と同様，胆道癌では根治治療後の再発率が高く，術後補助療法が必要と考えられているが，十分なエビデンスは確立していない。2002年，Takadaらは膵・胆道癌において，切除後MMCと5-FU併用療法(MF群)と切除単独群によるRCTを報告している[12]。胆管癌，乳頭部癌では生存に差はみられなかったが，胆嚢癌ではMF群で良好な長期生存率が得られ，特に非治癒切除例で有効性が示された。上述のように切除不能胆道癌ではGEMやS-1の有効性が認められつつあり，胆道癌診療ガイドラインではこれらを用いた術後補助療法の臨床試験を積極的に施行することが勧められている[4]。

膵　癌

1. 切除不能例に対する化学療法

GEMは，5-FUとの第Ⅲ相試験の結果QOLと生存期間に対する有効性が報告されて以来[13]，切除不能膵癌に対する標準治療として確立した。わが国でも膵癌診療ガイドラインにおいてGEM単独治療が推奨されている[3]。

その後，GEM単独治療の成績を超えるべく多くの新しい治療法の開発が行われてきた。そのなかでGEM＋erlotinibとGEM＋capecitabineの2つの併用療法がGEM単独に比べ有意に生存期間を延長したという結果が報告されている。

erlotinibの比較試験ではGEM単独のOS中央値が5.91ヵ月であったのに対し，GEM＋erlotinibのOS中央値は6.24ヵ月とその差はわずかであるが，GEM単独に比べ有意な生存期間の延長が得られた(ハザード比0.82，$p=0.038$)。GEM単独治療が確立した後，初めてエビデンスを示した治療

法である。しかし，間質性肺炎様症候群が2.5％にみられるなど重篤な有害事象も認めている[14]。コストや副作用を考慮するとそのまま標準治療として普及することは難しく，最適な対象を同定することが重要である。GEM＋erlotinib治療群をさらに詳細にみると皮疹が強く現れた患者群での生存期間が明らかに延長しており，皮疹の程度と治療効果が相関することが報告されている[14]。GEM＋erlotinib併用療法は米国，ヨーロッパで膵癌に適応承認が得られており，わが国でも同治療法の安全性と有効性を確認する治験が第Ⅱ相試験として実施された。erlotinibの有効性が期待できる対象を絞り込むことができれば膵癌の治療戦略の大きな柱になりうるものと考えられる。

GEM＋フッ化ピリミジン剤併用療法は，5-FUによりGEMの細胞内への取り込みが高まるなどから併用の意義が認められている。GEM＋フッ化ピリミジン剤併用のレジメンがいくつか試みられ，スイスで行われたGEM＋capecitabine併用療法では良好な成績を認めたものの，GEM単独に有意な上乗せ効果は示せなかった。しかし，英国で行われた比較試験では同併用療法において有意にGEM単独を上回る成績が得られており，今後の追試が待たれる。わが国ではS-1を中心に開発が行われてきた。遠隔転移を有する膵癌に対し，S-1単独でも奏効率37.5％，OS中央値9.2ヵ月と良好な成績が得られ[15]，GEM＋S-1併用療法では奏効率44.4％，OS中央値10.1ヵ月とさらに良好な成績が報告されている[16]。現在，GEM単独，S-1単独およびGEM＋S-1併用療法の3群による大規模な無作為化比較第Ⅲ相試験が行われている。

最近注目されているのは，分子標的薬を用いた治療法の動向である。先に述べたようにGEM＋erlotinib併用療法において，OSの差がわずかとはいえ有意差が示されたことから，他の分子標的薬に期待が集まっていた。なかでもbevacizumabとcetuximabは大腸癌などで有効性が示され，膵癌でもGEMとの併用による第Ⅱ相試験では良好な成績が得られていた。しかし，2007年のASCO会議でそれぞれ第Ⅲ相試験の結果が報告され，いずれもGEM単独を上回る成績が得られなかった。同ASCO会議で選択的VEGFR阻害薬であるaxitinibとGEM併用療法の無作為化比較第Ⅱ相試験が報告された[17]。GEM単独群34例，GEM＋axitinib併用群69例と症例数は少ないため有意差は認めないものの，OS中央値は単独群5.6ヵ月に対し，併用群

6.9ヵ月ハザード比0.74と後者で良好であった。現在，プラセボコントロールによるRCTが国際治験として実施されている。

2. 切除後補助化学療法

膵癌では根治切除後も高率に再発が認められ，早くから補助療法が検討されてきた。米国ではGastrointestinal Tumor Study Group（GITSG）で行われた無作為化比較試験に基づき化学放射線療法が標準治療として位置づけられている[18]。一方，ヨーロッパではEuropean Organisation for Research and Treatment of Cancer（EORTC）で行われた同様の試験において有意差が認められなかったことや，その後のEuropean Study Group for Pancreatic Cancer（ESPAC-1）の試験ではむしろ化学放射線療法は無治療群より予後不良であったことから化学療法が主流となっている[19]。わが国では診療ガイドライン上，ESPAC-1等の結果に基づいて5-FU based化学療法が推奨されているが，確立されたレジメンはないとされている[3]。またGEMの術後補助療法について，その延命効果は現時点で確定していないとしている[3]。

2007年，無治療とGEM化学療法による無作為化比較試験CONK-001試験の結果が報告された。主評価項目である無病生存期間が無治療群6.9ヵ月に対しGEM群13.4ヵ月と有意に良好であった（$p < 0.001$）こと，OSでは有意差を認めなかったものの（$p = 0.06$），5年生存率は無治療群11.5％，GEM群22.5％とでほぼ2倍の成績が得られたことから術後補助療法としてのGEMの有効性が示された[20]。

わが国ではこれまでMMC＋5-FU併用療法や5-FU＋CDDP併用療法と無治療との比較試験が行われているがいずれも有意な差は認められていなかった[14]。しかし厚生労働省がん研究小菅班においてCONK-001試験と同様のGEMと無治療との比較試験が行われ，無病生存期間で有意な延長を認めており，CONK-001と同様の結果が得られている。現在，静岡県立静岡がんセンターを始めとした多施設共同研究としてGEMとS-1による第Ⅲ相試験が行われており，結果が待たれる。

おわりに

肝・胆道・膵癌における化学療法は，この数年多くの臨床試験が行われ，

成果が上がってきている。しかしそれらの治療成績はまだまだ満足できるものではなく，今後より有効な治療法の開発と質の高い臨床試験の実施による検証が必要である。一方では，現在の最適な治療法として位置づけられた標準治療を適切に実施することも求められている。

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15. 肝がん治療の方向性 ～今後の治療法～

1）分子標的治療（ソラフェニブ）

肝細胞がんでは，切除手術，ラジオ波焼灼療法，肝動脈塞栓術など種々の有効な治療があるものの，それらの治療が適応とならない場合の有効な治療は，確立していませんでした。上記のような治療の効果が期待できない場合は通常，抗がん剤を用いた化学療法が行われますが，肝細胞がんは従来の抗がん剤が効きにくいがんと考えられてきました。

近年，がん細胞の増殖や進行に関わるメカニズムが明らかになってくると，鍵となる特定のポイント（分子）をターゲット（標的）として，がんの進行を抑える治療が進められてきました。いわゆる分子標的薬の開発です。分子標的薬の一つであるソラフェニブは複数のポイントを阻害する新しい薬で，さまざまながんで有効性が確認されつつあります。最近，肝細胞がんでも優れた効果が証明されたことから，今後の肝細胞がんの治療を大きく変える薬として期待されています（2008 年 12 月現在，承認申請中）。

分子標的薬の特徴

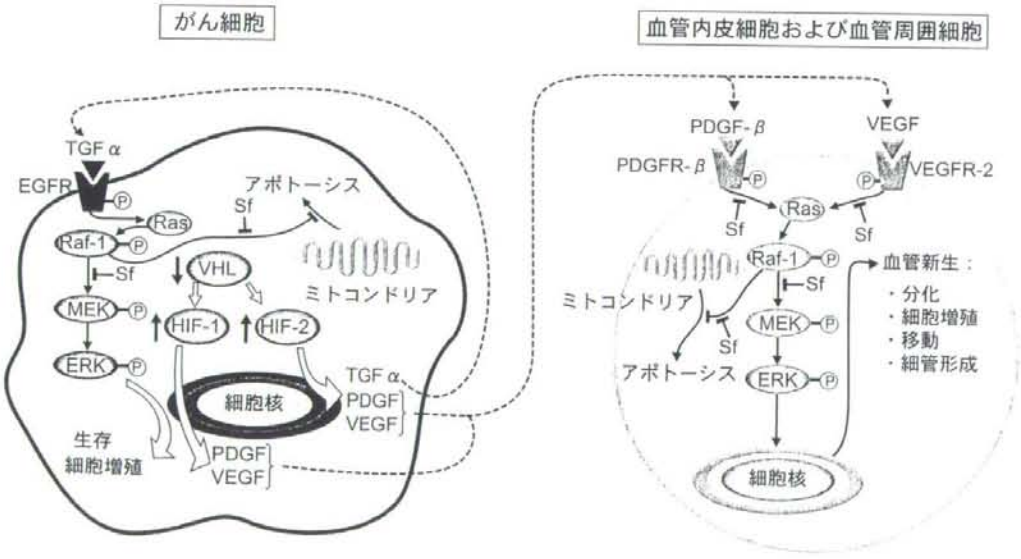
従来の抗がん剤が，自然界を含め多くの物質からがんに効くものを探して「くすり」として開発していくのに対し，分子標的薬は，がん細胞の増殖や転移などに関わる特定の部位をターゲットに開発されます。従来の抗がん剤は細胞の合成そのものを阻害し，細胞を殺す作用が主体で，一般的に副作用も強いのが特徴です。一方，分子標的薬は，腫瘍細胞に強く発現している細胞増殖に関する因子を抑制することで，腫瘍の増殖を抑えることが主な作用であり，副作用は概して強くない傾向があります。しかし，手足皮膚反応など従来の抗がん剤ではあまりみられない副作用が出ることがあり，それらの対策も大切です。

	従来の抗がん剤	分子標的薬
創薬	自然界などの多くの候補から，抗腫瘍効果を持つものを探す	がん細胞の増殖メカニズムに基づき，特定の分子をターゲットに合成する
作用機序	DNA 合成阻害，代謝拮抗など	細胞増殖シグナル阻害，血管内皮増殖阻害など
特徴	殺細胞性	細胞増殖抑制性
有効性の評価	病変の縮小	増悪までの期間
主な特徴的副作用	骨髄毒性，消化器毒性，腎毒性，脱毛など	皮疹，手足皮膚反応，間質性肺炎，高血圧など

ソラフェニブの作用機序

がん細胞では，さまざまな因子が細胞の外から作用して，細胞の中にシグナルが伝わり，増殖や転移につながっていきます。細胞の増殖は正常の細胞でも起こっていますが，がん細胞で特に強く発現している因子やシグナルがあり，そこを抑えることでがんの増殖や転移を抑制すると考えられます。また，がん細胞自体ががんの周囲の細胞に作用して豊富な血管新生が起こり，さらにがんの増殖が早まることがわかっています。この血管新生を阻害することも，がんの進行を抑える重要なポイントとなっています。

細胞の中の重要な増殖シグナル伝達因子の一つに「Raf」という分子があり，肝細胞がんを含めたいくつかのがんでは，そのはたらきが強くなっています。ソラフェニブはこの「Raf」のはたらきを阻害して，がんの増殖を抑制します。またがん細胞からは，周囲の血管内皮細胞や血管周囲細胞を刺激して血管新生を活発にする物質（VEGF，PDGF など）がたくさん放出されています。ソラフェニブは，血管内皮細胞の表面にあるそれらの物質の受容体（VEGFR，PDGFR）を阻害することで血管新生を抑制し，がんの進行を妨げる作用も持っています。ソラフェニブのように，複数のポイントを阻害することでがんの増殖を抑制する薬を，マルチキナーゼ阻害剤といいます。



ソラフェニブの作用機序

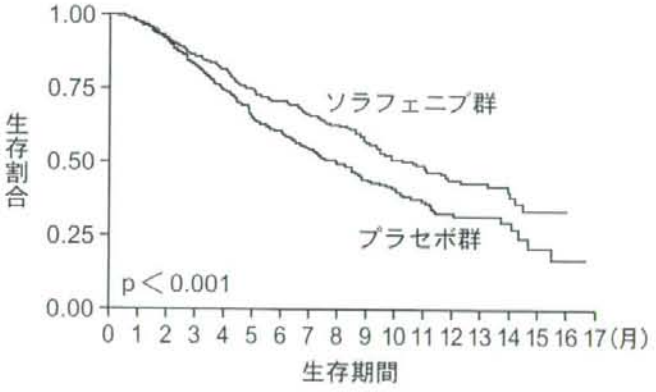
Sf：ソラフェニブ
アポトーシス：プログラムされた細胞死
（Gollob JA, et al：Semin Oncol 33：392-406, 2006 を基に作成）

ソラフェニブの適応と治療成績

肝がんには多くのタイプが含まれます。肝臓自体から発生した原発性肝がんと，他の臓器のがんが転移してできた転移性肝がんがあり，さらに原発性肝がんにもいくつかタイプがあります。ソラフェニブは，原発性肝がんの中でも一番数の多い肝細胞がんに適応となります。

肝細胞がんでは，切除手術やラジオ波焼灼療法，肝動脈塞栓術など，肝臓の中の病変を治療するさまざまな方法(局所治療)が確立しています。ソラフェニブはそれら局所治療の適応がない病状で臨床試験が行われ，有効性が確認されました。つまり，これまで有効な治療がなかった状態，特に肝臓の外に転移がある場合や局所治療が無効な場合に用いられます。

そのような患者さんを対象に，欧州を中心とした大規模な臨床試験が行われました。ソラフェニブを内服する群とプラセボ(薬の成分の入っていないもの)群にランダムに振り分けて治療効果を比較する，ランダム化比較試験です(SHARP試験と名前が付けられました)。その結果，ソラフェニブ群で44%の生存期間の延長効果が確認され，統計学的に有意な有効性が認められました。下図はSHARP試験での生存曲線です。プラセボ群よりソラフェニブ群の曲線のほうが全体に上にきていて，良好な治療成績が得られています。



進行肝細胞がん患者におけるソラフェニブとプラセボによるランダム化比較試験（SHARP 試験）の生存曲線
(Llovet JM, et al : N Engl J Med 359 : 378-390, 2008 より改変引用)

ただし，ソラフェニブは肝細胞がんに有効性が確認されたとはいえ，腫瘍を縮小させるというよりも，増殖を抑え進行を遅らせる効果が主体です。つまり，ソラフェニブで病変が完全に消えるわけではありませんので，特徴を活かした使い方が大切です。また，これまでの使用経験は肝機能良好な患者さんがほとんどであり，肝機能が低下した患者さんでの安全性や有効性は十分検討されていません。肝機能を十分チェックした上で適応を決める必要があります。

ソラフェニブの副作用

わが国でもSHARP試験と同様に，進行肝細胞がんの患者さんを対象として安全性や薬物動態をみる臨床試験（第Ⅰ相試験）が行われ，十分な忍容性（副作用に耐えうる程度）が認められています。しかし，皮疹，手足皮膚反応，下痢，高血圧などさまざまな副作用が認められています。

副作用は個人差が大きく，必ずしもすべての患者さんに現れるとは限りませんが，十分な注意が必要です。早めに副作用に対する治療薬を使ったり，重い場合はソラフェニブの内服を休んだりすることで対応します。特に，ソラフェニブで特徴的な手足皮膚反応では，窮屈な靴や身体に密着・圧迫する衣服を避けるなどの予防も大切です。

ソラフェニブの主な副作用

主な副作用	SHARP試験（299例）		日本での第Ⅰ相試験（27例）	
	全グレード	グレード3・4	全グレード	グレード3・4
高血圧	**5%**	**2%**	**19%**	**19%**
疲労	22%	4%	4%	0
発熱	−	−	15%	0
体重減少	9%	2%	30%	0
手足皮膚反応	**21%**	**8%**	**44%**	**7%**
皮疹	**16%**	**1%**	**56%**	**7%**
脱毛	14%	0	19%	0
皮膚乾燥	8%	0	11%	0
掻痒感	8%	0	30%	0
食欲低下	14%	＜1%	22%	0
悪心	11%	＜1%	0	0
口内炎	−	−	11%	0
下痢	**39%**	**8%**	**56%**	**4%**
腹痛	8%	2%	−	−
出血	7%	＜1%	0	0

−：記載なし
グレード3，4は程度の重い副作用を示します。
赤字の項目はソラフェニブで頻度の高い，特徴的な副作用です。
（Llovet JM, et al : N Engl J Med 359 : 378-390, 2008 およびFuruse J, et al : Cancer Sci 99 : 159-165, 2008より改変引用）

（古瀬純司）

HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: Preclinical study using mice

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Abstract. We previously reported that glypican-3 (GPC3) is uniquely overexpressed in human hepatocellular carcinoma and melanoma and that it is an ideal tumor antigen for immunotherapy in mouse models. We recently identified both HLA-A24 (*A*2402*) and H-2K^d-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2 (*A*0201*)-restricted $GPC3_{144-152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs). The present study was a preclinical study in a mouse model that was conducted in order to design an optimal schedule for clinical trial of GPC3-derived peptide vaccine. When BALB/c mice were intradermally vaccinated at the base of the tail with K^d-restricted $GPC3_{298-306}$ peptide mixed with incomplete Freund's adjuvant (IFA), the peptide-specific CTLs were induced. But the peptide alone could not induce peptide-specific CD8$^+$ T cells. Furthermore, proteomic analyses showed that IFA protected the peptide against degradation in the human serum. Peptide-reactive CTLs were induced by peptide vaccine in a dose-dependent manner. In addition, at least two vaccinations with a single dose >10 μg were needed for the induction of $GPC3_{298-306}$-specific CTLs. But repeated vaccination with a lower dose of $GPC3_{298-306}$ did not induce peptide-specific CTLs. Similarly, induction of an Ag-specific immune response by HLA-A2 $GPC3_{144-152}$ depended on the dose administered. The results of this study suggested that IFA is one of the indispensable adjuvants for peptide-based immunotherapy, and that the immunological effect of peptide vaccines depends on the dose of peptide injected.

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Abbreviations: BM-DC, bone marrow-derived dendritic cell; mAb, monoclonal antibody; M/W, molecular/weight

Key words: cancer immunotherapy, GPC3, peptide vaccine, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide, especially in Asian and Western countries (1). Despite advances in diagnosis and treatment, the overall survival of patients with HCC has not significantly improved in the last two decades (2). The effective treatments currently available are only indicated in a relatively small proportion of early stage cases. When patients presents with clinical manifestations of HCC, the tumor is usually advanced, and there are few treatment options. Many HCC patients have type B or C hepatitis or cirrhosis, so patients treated surgically or by other therapies are also at high risk for recurrence. Furthermore, the liver function of such patients is often very poor, so treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor and new therapies for cancer development and recurrence, i.e., adjuvant therapy, are urgently needed.

We previously reported that glypican-3 (GPC3), glycosylphosphatidylinositol (GPI)-anchored membrane protein, is specifically overexpressed in human HCC and melanoma, and that among normal tissues it is slightly expressed in placenta and embryonic liver (3). We found that GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several studies with mice (4,5). In addition, we identified CTL epitope peptides: HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) (6). To design the schedule for the phase I clinical study of these GPC3-derived peptide vaccines, many factors need to be taken into consideration: the adjuvant, dosage, number of doses, vaccination interval, etc. Many investigators have reported various vaccination schedules (7,8). There is no world-wide consensus concerning the schedule to use for administration of peptide

vaccines. In this study, we attempted to identify a more effective vaccine regimen that would induce a strong cell-mediated immune response. Ten years ago Salgaller *et al* reported that they did not observe any dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). The results of our present study, however, showed dose-dependency in the immunizing effect of a peptide vaccine.

Materials and methods

Mice. Female BALB/c mice at 6-8 weeks of age were obtained from Japan SLC (Hamamatsu, Japan) or Charles River Laboratories. *HLA-A2.1* (HHD) Tgm; *H-2D*$^{b-/-}$*h2m*$^{-/-}$ double knockout mice transfected with a human h2m-*HLA-A2.1* (a1 a2)-*H-2D*^{b} (a3 transmembrane cytoplasmic) (HHD) monochain construct gene were prepared in the Department SIDA-Retrovirus, Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, France (10), and kindly provided by Dr F.A. Lemonnier. The mice were maintained under specific-pathogen-free conditions. The mouse experiments were approved by the Animal Research Committee of the National Cancer Center Hospital East.

Cells lines. A subline of BALB/c-derived colorectal adenocarcinoma cell line Colon 26, C26 (C20) (11) was provided by Dr Kyoichi Shimomura (Astellas Pharmaceutical Co., Tokyo, Japan). Colon 26/GPC3 (C26/GPC3) is an established stable GPC3-expressing cell line (4). RMA-HHD cells were kindly provided by Dr Masanori Matsui of Saitama Medical School, Saitama, Japan. A human ß2m-HLA-A2.1 (α1α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) monochain construct was transfected into RMA lymphoma cells [transporter associated with antigen presentation (TAP) positive] to establish RMA-HHD cells (10). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS. To obtain GPC3-expressing RMA-HHD (RMA-HHD-GPC3) cells, RMA-HHD cells were transfected with pCAGGS-GPC3-internal ribosomal entry site (IRES)-puromycin-resistant gene with Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA), selected with puromycin, and then subjected to cloning by limiting dilution in drug-free medium in 96-well culture plates (12,13). Dendritic cells were obtained from bone marrow cells (BM-DCs) as described previously (4). Irradiated BM-DCs pulsed with peptide were used for *in vitro* CTL culture or as target cells for Elispot assays.

Vaccination. HLA-A24- and K^{d}-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) were dissolved in 7% $NaHCO_3$ and the solution was diluted with saline. For peptide vaccination, mice were intradermally injected at the base of the tail with peptide solution emulsified in incomplete Freund's adjuvant (IFA). Different doses of peptide were administered at 7-day intervals, and mice were sacrificed to obtain inguinal lymphocytes and spleen cells seven days after the final vaccination.

IFN-γ Elispot assays. Female BALB/c mice were intradermally vaccinated with $GPC3_{298-306}$ or $GPC3_{144-152}$/IFA. Their inguinal lymphocytes were stimulated with peptide-pulsed BM-DCs *in vitro* for five days. The proportion of cells producing IFN-γ against target cells (BM-DCs pulsed with or without GPC3 peptide) was assessed by an Elispot assay as described previously (14). The spots were automatically counted and subsequently analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

Analysis of peptide degradation. $GPC3_{298-306}$ was mixed with human serum, and the solution was applied to the surfaces of a Q10 (strong anion exchange) ProteinChip (Bio-Rad Laboratories, CA). We sequentially examined the solution with a SELDI-TOF mass spectrometer (Bio-Rad). Female BALB/c mice were intradermally vaccinated with $GPC3_{298-306}$/IFA at the base of the tail. A week later, we collected the residual peptide vaccine at the base of the tail into buffer in a tube, and the tube was centrifuged at 4˚C at 10,000 rpm for 15 min. The supernatant was applied to a ProteinChip and the surface of the chip was examined with the spectrometer.

Induction of GPC3-specific CTLs and cytotoxicity assay. Mice were intradermally vaccinated twice with 50 μg GPC3 peptide/IFA 7 days apart. Seven days after the second vaccination, inguinal lymph nodes were excised and the lymphocytes were cultured in 24-well culture plates ($5x10^6$ per well) with GPC3 peptide-pulsed BM-DCs ($1x10^5$ per well) in RPMI medium supplemented with 10% horse serum, recombinant human interleukin (IL)-2 (100 units/ml), and 2-mercaptoethanol (50 μmol/l). After culture for 5 days, the cells were recovered and analyzed for their cytotoxic activity against target cells with the TERASCAN VPC system (Minerva Tech) as previously described (15). Briefly, C26, C26/GPC3, RMA-HHD and RMA-HHD-GPC3 cells were used as target cells and labeled with calcein-AM solution for 30 min at 37˚C. The labeled cells were washed three times and distributed to the 96-well culture plate ($1x10^4$ per well), and they were then incubated with effector cells for 5-6 h. Fluorescent intensity was measured before and after the 5-6-h culture, and Ag-specific cytotoxic activity was calculated by using this formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100. In some experiments, CD8^{+} T cells were isolated from effector cell preparations with a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Positively selected CD8^{+} T cells were 95% pure as determined by flow cytometry.

Histologic and immunohistochemical analysis. Mice were injected twice with GPC3 peptide vaccine, and seven days later tumor cells were subcutaneously implanted in their shaved backs. Seven days after the tumor challenge, frozen sections of tumor tissue were prepared. The frozen tissue sections were immunohistochemically analyzed using monoclonal antibody (mAb) specific for CD4 (L3T4; BD PharMingen, San Diego, CA) or CD8 (Ly-2; BD PharMingen) as described previously (4).

Statistical analysis. The 2-tailed Student's t-test was used to determine the statistical significance of differences in the

data obtained by ELISPOT assay. P<0.05 was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

IFA is an effective adjuvant for the peptide vaccine that induced strong immune responses and maintained the stability of the peptide. We attempted to verify whether emulsions of GPC3 peptide in IFA could induce a peptide-specific immune response in mouse models. The results showed that only GPC3 peptide emulsified in IFA elicited a T-cell-mediated immune response, whereas vaccination with peptide alone failed to induce any detectable immune response (Fig. 1A). In addition, we investigated the stability of GPC3 peptide alone in human serum, with the Surface Enhanced Laser Desorption/Ionization SELDI system (Bio-Rad). We applied the sample of GPC3 peptide mixed with human serum to a ProteinChip and detected the peak of GPC3 peptide. The peak value of $GPC3_{298-306}$ in buffer was about 1,000, whereas the peak of $GPC3_{298-306}$ in serum had decreased to 200, 3 min after mixing it with the serum (Fig. 1B). This finding indicated that GPC3 peptide was immediately degraded in serum. Moreover, we collected the white residue of peptide/IFA emulsions that were still present at the base of the tail of the vaccinated mice, and after applying the peptide/IFA emulsions to a ProteinChip, quantified the peak of $GPC3_{298-306}$. Mass spectrometric analysis demonstrated that the peptide was still present in a stable form in the peptide/IFA emulsions (Fig. 1C). IFA not only induced a potent immune response, but protected the peptide from various enzymes in the serum.

Dose-dependent effects of GPC3-derived peptide vaccine emulsified in IFA. Next we examined whether a more peptide-specific response was induced, when a higher dose of peptide was used. The proportion of peptide-specific CTLs among $5x10^4$ $CD8^+$ T cells was evaluated by IFN-γ Elispot assays, when mice were vaccinated with $GPC3_{298-306}$ K^d-restricted peptide doses of 5, 10, 20, or 50 μg. Peptide-specific $CD8^+$ T cell responses were observed when vaccinated with $GPC3_{298-306}$ K^d-restricted peptide doses above 10 μg (Fig. 2A). Additionally, as the peptide dose increased, peptide reactive CTLs were detected more frequently (Fig. 2A). We vaccinated A2 Tg mice with HLA-A2-restricted $GPC3_{144-152}$ peptide in the same manner. The results indicated that the higher doses of the peptide induced a greater peptide-specific immune response (Fig. 2B). We therefore, concluded that the higher the dose of peptide injected, the more peptide-specific $CD8^+$ T cells were induced. But Elispots by vaccinations of >5 μg $GPC3_{144-152}$ been seen to reach a plateau level.

Marked infiltration of subcutaneous tumor tissue by $CD8^+$ T cells in mice vaccinated with the 50 μg dose of GPC3 peptide. Immunohistochemical analysis of the tumor tissue specimens showed more intense infiltration by $CD8^+$ T cells, but not by $CD4^+$ T cells, in and/or around C26/GPC3 (Fig. 3D) or RMA-

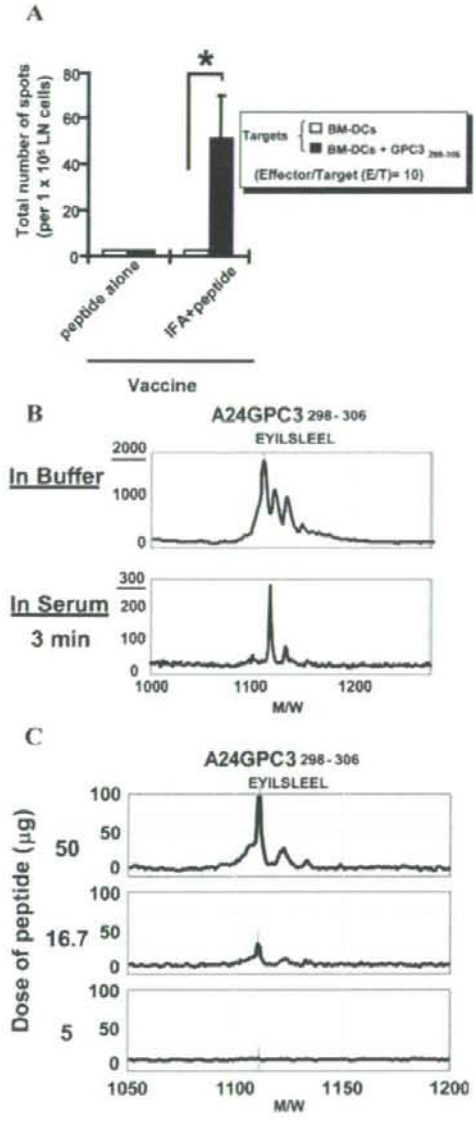

Figure 1. IFA is an appropriate adjuvant for the peptide vaccine. (A) The $GPC3_{298-306}$-specific immune response was induced by peptide emulsified in IFA. Female BALB/c mice were vaccinated twice by intradermal injection of 50 μg of $GPC3_{298-306}$ (EYILSLEEL) with IFA. Seven days after the second injection, inguinal lymph node cells were isolated and cultured with $GPC3_{298-306}$-pulsed BM-DCs for 5 days. The cells were recovered, and their Ag-specific activity was analyzed by IFN-γ Elispot assays against BM-DCs pulsed or not pulsed with $GPC3_{298-306}$. *P<0.05, statistically significant difference in response. (B) Stability of $GPC3_{298-306}$ (EYILSLEEL) in human serum. We measured $GPC3_{298-306}$ in the human serum 3 min after the mixture of $GPC3_{298-306}$ and human serum by SELDI-TOF mass spectrometry. The discriminating peaks of M/W 1111 represent $GPC3_{298-306}$. When GPC3 peptide was mixed with human serum, the peptide was immediately degraded by various proteases. (C) Detection of GPC3 peptide in emulsions collected from vaccinated mice. BALB/c mice were intradermally injected at the base of the tail with $GPC3_{298-306}$ emulsified in IFA. A week later we collected peptide/IFA emulsions at the base of the tail and measured GPC3 peptide contained in the emulsions. When injected with 50 μg of peptide, GPC3 peptide was detected clearly. Data are representative of 3 independent experiments with similar results in (A-C).

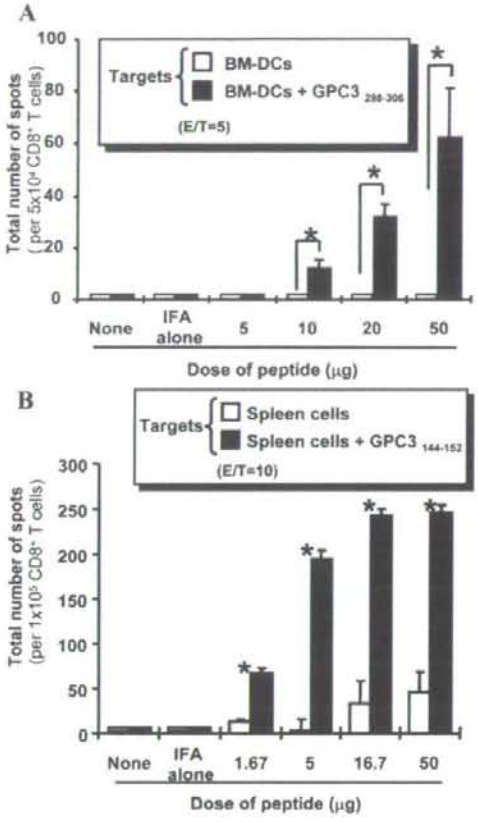

Figure 2. The higher the dose of peptide injected, the more peptide-specific $CD8^+$ T cells were induced. Groups of mice were vaccinated twice at 7-day intervals with one of the dose levels of H-$2K^d$-restricted $GPC3_{298-306}$ peptide (A) or HLA-A2-restricted $GPC3_{144-152}$ peptide (B) emulsified with IFA (each group; n=3). Inguinal lymphocytes were restimulated *in vitro* with each GPC3 peptide-pulsed BM-DCs for 5 days. The recovered cells were sorted to the $CD8^+$ T cells fraction by MACS and IFN-γ Elispot assays were performed. Peptide-specific $CD8^+$ T cells were induced dose-dependently. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

HHD-GPC3 (Fig. 3H) tumor tissue of mice vaccinated with the 50 μg dose of GPC3 peptide than with IFA alone, and the 1.67 μg dose of GPC3 peptide (Fig. 3). This phenomenon was also observed in metastatic inguinal lymph nodes tissues (data not shown). These results also suggest that the higher the dose of peptide, the more peptide-specific $CD8^+$ T cells were induced and infiltrated the GPC3-expressing tumor. However, 1.67 μg dose of $GPC3_{144-152}$ had seemed to induce a few peptide-specific $CD8^+$ T cells, which corresponded to the result shown in Fig. 2B.

A second vaccination is needed to induce a peptide-specific response. Next we attempted to determine how many vaccinations were required to induce a peptide-specific immunological response. BALB/c mice were vaccinated with a 1.67, 5, 16.7, 50 μg dose of peptide once a week for 1-4 weeks, respectively. A single vaccination did not elicit a peptide-specific immune response at any of the

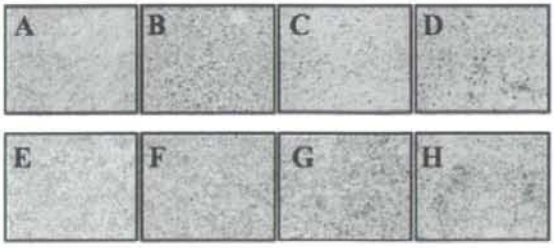

Figure 3. Infiltration by $CD8^+$ T cells around and into subcutaneous C26/GPC3 (A-D) or RMA-HHD-GPC3 (E-H) tumor tissue is shown. We estimated infiltration by $CD8^+$ T cells immunohistochemically; no treatment (A and E), IFA (B and F), 1.67 μg (C), 50 μg (D) of $GPC3_{298-306}$, 1.67 μg (G), 50 μg (H) of $GPC3_{144-152}$. In mice treated with 50 μg GPC3 peptide, a larger number of $CD8^+$ T cells had clearly infiltrated into and/or around the tumor (D and H). But even in 1.67 μg dose of $GPC3_{144-152}$, a few peptide-specific $CD8^+$ T cells were induced (original magnification, x200).

$GPC3_{298-306}$ dose levels (Fig. 4A). Induction of a peptide-specific T-cell response required at least two vaccinations and >16.7 μg dose of peptide, and no expansion of peptide-specific T cells occurred after repeated vaccinations with lower doses of peptide (Fig. 4B-D). We also compared immunological responses induced by two and five vaccinations, with 1.67 and 50 μg doses of HLA-A2 $GPC3_{144-152}$, but the same as with HLA-A24 $GPC3_{298-306}$, five vaccinations did not increase a peptide-specific response (Fig. 5).

Cytotoxity of $CD8^+$ T cells primed with GPC3 peptide vaccines. We analyzed the cytotoxity of $CD8^+$ T cells primed with GPC3 peptide vaccines. Their killing activity against target cells that expressed or did not express GPC3 was analyzed. The effector cells primed with the GPC3 vaccines showed a significantly higher killing activity against C26/GPC3 cells than against C26 cells, and significantly higher killing activity against RMA-HHD-GPC3 cells than against untransfected RMA-HHD cells (Fig. 6). These results suggest that the $CD8^+$ T cells induced by GPC3 peptide vaccinations have cytotoxic activity against tumor cells that express GPC3 naturally.

Discussion

The stability of antigens and the immunogenicity of ISA 720 based on Western blot experiments (16) have been verified, and in the present study we showed that IFA is one of the indispensable adjuvants for peptide vaccines.

We previously reported that vaccination with $GPC3_{298-306}$ peptide-pulsed BM-DCs induced complete rejection of a C26/GPC3 tumor challenge in a mouse model (4), but in the present study, C26/GPC3 tumors in a prophylactic model were not rejected after two intradermal vaccinations with GPC3 peptide/IFA at the base of the tail even though $CD8^+$ T cells by GPC3 peptide vaccine was demonstrated by immunological and immunohistological analysis (data not shown). Comparison of the capacity of peptide-pulsed BM-DCs vaccine to induce peptide-specific CTLs with the

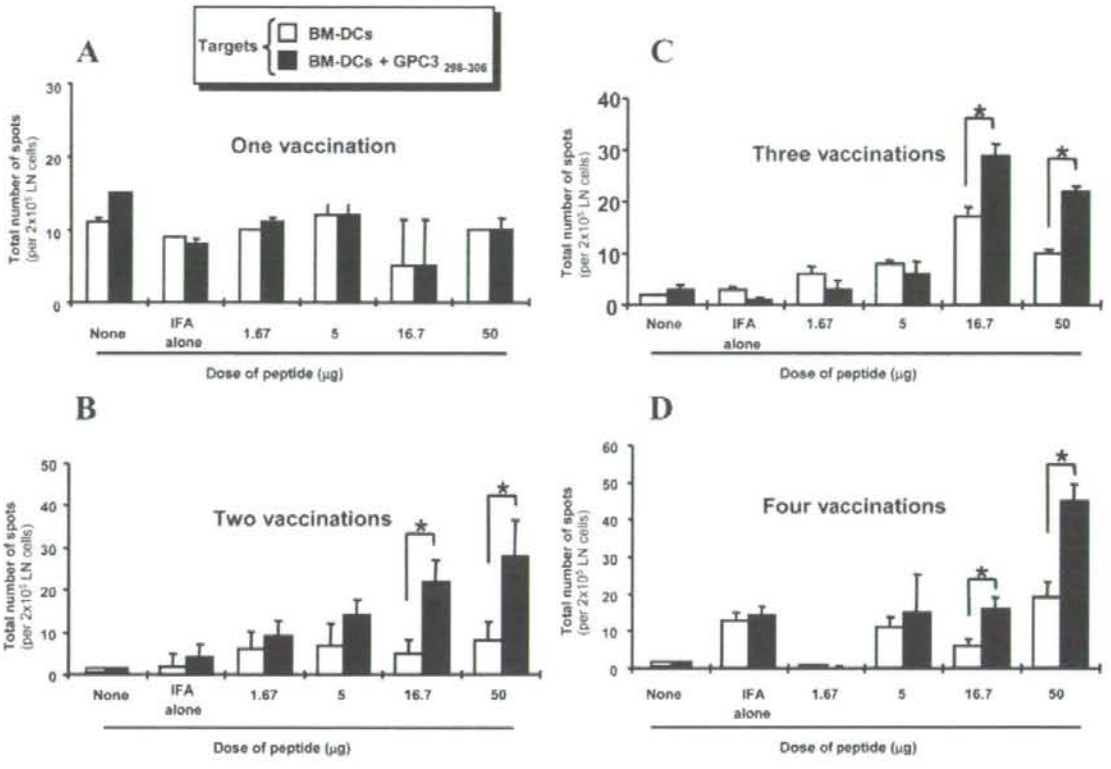

Figure 4. A second vaccination is needed to induce peptide-specific T cells. The immune responses to one (A), two (B), three (C) and four (D) vaccinations with each dose of peptide are shown. BALB/c mice (each group: n=3) were vaccinated with 1.67, 5, 16.7, or 50 μg $GPC3_{298-306}$. Seven days after the final vaccination, bilateral inguinal LNs were excised. Each lymphocyte was restimulated *in vitro* with $GPC3_{298-306}$ peptide-pulsed BM-DCs for 5 days, and IFN-γ Elispot assays were then performed against BM-DCs pulsed or not pulsed with $GPC3_{298-306}$ to count $GPC3_{298-306}$ peptide-specific CTLs. Data are representative of 3 independent experiments with similar results (A-D).

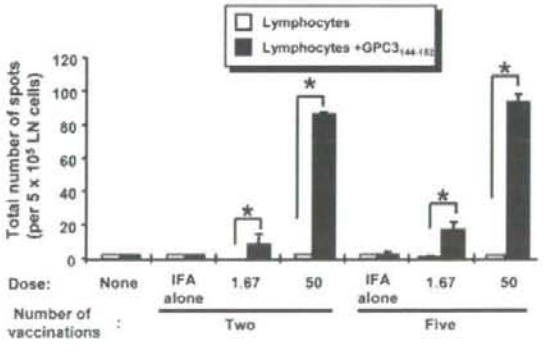

Figure 5. Comparison of immune responses after two or five vaccinations with a 1.67 μg or 50 μg dose of $GPC3_{144-152}$. A2 Tg mice (n=3) were vaccinated with 1.67 μg or 50 μg $GPC3_{144-152}$ in the same manner in Fig. 3, but the IFN-γ Elispot assay was performed using whole lymph node cells without *in vitro* culture. Data are representative of 3 independent experiments with similar results. *P<0.05, difference in response was statistically significant.

capacity of peptide/IFA vaccine by *in vitro* IFN-γ Elispot assays demonstrated that peptide-pulsed BM-DCs vaccine induced more peptide-specific CTLs (data not shown). There have been few reports of induction of tumor regression *in vivo* by peptide vaccine. Pilar *et al* recently reported finding that a combination of peptide vaccine and CpG induced stronger anticancer responses not only in a prophylactic model, but also in a therapeutic model. They reported in the same study that vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses (17). Although other adjuvants, including CpG, were not effective in the present study (data not shown), peptide/IFA with CpG may be effective. Further study is needed.

The results of the present study showed that at least two vaccinations were necessary to elicit immunological effects. A comparison between HLA-A2-restricted $GPC3_{144-152}$/IFA and K^d-restricted $GPC3_{298-306}$ showed that $GPC3_{144-152}$ induced more peptide-specific CTLs at a lower dose. Moreover, HLA-A2-restricted $GPC3_{144-152}$ specific CTLs were induced without *in vitro* stimulation with $GPC3_{144-152}$ peptide. That may have been attributable to the difference in mouse strain. It is usually said that C57BL/6 and BALB/c mice are a prototypical Th1-type strain and a prototypical Th2-type mouse strain, respectively (18,19) and the difference in genetic background seemed to affect their susceptibility to each of the peptide vaccines.

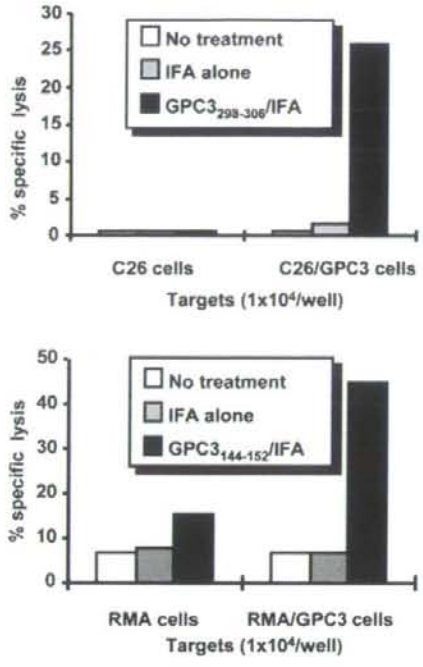

Figure 6. Cytotoxity of $CD8^+$ T cells primed with GPC3 peptide vaccine. BALB/c or C57BL/6 mice (n=2-3) were vaccinated with IFA alone or 50 μg peptide/IFA in the same manner as described above. The inguinal LN cells were cultured with $1x10^5$ peptide-pulsed BM-DCs for 5 days. The cells obtained were sorted to the $CD8^+$ T cell fraction with microbeads. Cytotoxic assays were performed with the cells to evaluate their capacity to kill $1x10^4$ C26, C26/GPC3, RMA-HHD, or RMA-HHD-GPC3 cells. Data are representative of 3 independent experiments with similar results.

In 1996, Salgaller *et al* reported that they did not detect dose dependency between 1 and 10 mg in the capacity of gp100 peptide to enhance immunogenicity in humans (9). A dose-dependent effect of peptide vaccine was shown in the present study. We are conducting a phase I clinical trail of GPC3-derived peptide vaccine in patients with advanced HCC at National Cancer Center Hospital East, and we are waiting for the results to determine whether a dose-dependent effect of peptide/IFA was shown in humans, the same as in the mouse model.

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HLA-A2-restricted CTL epitopes of a novel lung cancer-associated cancer testis antigen, cell division cycle associated 1, can induce tumor-reactive CTL

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Toward the development of a novel cancer immunotherapy, we have previously identified several tumor-associated antigens (TAAs) and the epitopes recognized by human histocompatibility leukocyte (HLA)-A2/A24-restricted cytotoxic T lymphocyte (CTL). In this study, we tried to identify a TAA of lung cancer (LC) and its HLA-A2 restricted CTL epitopes to provide a target antigen useful for cancer immunotherapy of LC. We identified a novel cancer testis antigen, cell division cycle associated gene 1 (CDCA1), overexpressed in nonsmall cell LC using a cDNA microarray analysis. The expression levels of CDCA1 were also increased in the majority of small cell LC, cholangiocellular cancer, urinary bladder cancer and renal cell cancers. We used HLA-A2.1 transgenic mice to identify the HLA-A2 (*A*0201*)-restricted CDCA1 epitopes recognized by mouse CTL, and we investigated whether these peptides could induce CDCA1-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A2-positive donors and a NSCLC patient. Consequently, we found that the $CDCA1_{65-73}$ (YMMPVNSEV) peptide and $CDCA1_{351-359}$ (KLATAQFKI) peptide could induce peptide-reactive CTLs in HLA-A2.1 transgenic mice. In HLA-A2$^+$ donors, *in vitro* stimulation of PBMC with these peptides could induce peptide-reactive CTLs which killed tumor cell lines endogenously expressing both HLA-A2 and CDCA1. As a result, CDCA1 is a novel cancer-testis antigen overexpressed in LC, cholangiocellular cancer, urinary bladder cancer and renal cell cancers, and CDCA1 may therefore be an ideal TAA useful for the diagnosis and immunotherapy of these cancers.

Key words: tumor immunology; cancer testis antigen; CDCA1; CTL; HLA-A2

Lung cancer (LC), especially nonsmall cell lung cancer (NSCLC), is one of the most common cancers in the world, and more than 1 million people are killed by LC annually. The most common cause of cancer death is LC among men and women aged 60 years and older.[1]

Currently, new combination therapies are prescribed for patients with advanced NSCLC, but LC is relatively resistant to the currently available chemotherapy and radiotherapy regimens.[2] Most current regimens for NSCLC provide a limited survival benefit and are often considered to be ineffective or excessively toxic.[3]

Recently, the presence of lymphocytic infiltrates in murine and human tumors or generation of cytotoxic T lymphocytes (CTLs) recognizing lung tumor antigens suggest that an immune reaction could potentially help to eliminate tumor cells.[2] Adoptive immunotherapy using *in vitro* expanded tumor antigen-specific $CD8^+$ CTLs has been considered as a feasible therapy for *in vivo* eradication of tumors.[4] There have been reports on the clinical efficacy of immunotherapy for advanced cancer, but little clinical data have been reported in cases of advanced NSCLC.[5–7]

Tumor-associated antigens (TAAs) are proteins known to be overexpressed in and broadly distributed among malignant cells of various origins.[8,9] The molecular identification and characterization of expressed TAAs has rapidly evolved because of the availability of new technologies. Many TAAs in certain human malignancies were identified using methods of cDNA expression cloning.[10–12]

Recently, cDNA microarray technologies have been developed and the systematic analysis of the expression levels of thousands of genes is an effective method for the identification of TAAs overexpressed in cancer tissues.[13] We used a genome-wide expression profile analysis of LCs with the cDNA microarray containing 27,648 genes and investigated the biological and clinicopathological significance of the respective gene products.[13] This systematic approach revealed that cell division associated 1 (CDCA1) was frequently overexpressed in various histologic types of LC but not in normal adult tissues including lung tissues, except normal testis, and that CDCA1 was essential for growth or survival of LC cells.[13]

The protein CDCA1 has a specific function at the kinetochores to stabilize microtubule attachment in Hela cells. CDCA1 is a part of a molecular linker between the kinetochore attachment site and the tubulin subunits within the lattice of the attached plus ends. Therefore, the depletion of CDCA1 results in a strong prometaphase blockade with an active spindle checkpoint and it causes aberrant chromosome segregation. These CDCA1-suppressed cells displayed reduced tension at kinetochores of chromosomes.[14,15] CDCA1 was one of cell cycle-associated genes that was coexpressed with known cell cycle genes, including cell division cycle 2 (CDC2), cyclin, topoisomerase II and others.[16] On the other hand, CDCA1 is involved in the process of pulmonary carcinogenesis. The NSCLC patients with highly CDCA1 expressing cancerous tissue showed poorer prognosis in comparison to patients with low CDCA1 expression.[13] Therefore, we have considered that CDCA1 may prove to be applicable as a candidate target for cancer immunotherapy.

The gene frequency of human histocompatibility leukocyte (HLA)-A2 (A*0201) is high among various ethic groups, includ-

Abbreviations: ADC, adenocarcinoma; BM-DCs, bone marrow-derived dendritic cell; CDCA1, cell division cycle associated 1; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IL, interleukin; LC, lung cancer; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcription-PCR; SqCC, squamous cell carcinoma; TAAs, tumor-associated antigens; Tgm, transgenic mice.

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ing Asians, Africans, Afro-Americans and Caucasians.[17] It is suggested that the HLA-A2-restricted and CDCA1-derived CTL epitopes might be very useful for the immunotherapy of many patients with NSCLC all over the world. In this study, we identified human CDCA1-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 restricted epitope peptides could induce CDCA1-reactive CTLs from the peripheral blood mononuclear cells (PBMCs) of healthy donors.

Material and methods

Mouse

HLA-A2.1 (HHD) Tgm; H-2D$^{-/-}$ β2m$^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1, α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were a generous gift from Dr. F.A. Lemonnier of the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France.[18,19]

cDNA microarray and acquisition of data

A genome-wide cDNA microarray was fabricated by the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, containing 27,648 cDNAs selected from the UniGene Database (build No. 131) of the National Center for Biotechnology Information. Briefly, the microarray system containing 27,468 cDNAs was established previously.[20] Cancer cells were selectively collected from the preserved samples using laser microbeam microdissection (LMM) method.[21] Extraction of total RNA and T7-based amplification were performed as described previously. As a control probe, normal human lung poly(A) RNA (BD Biosciences Clontech, Palo Alto, CA) was amplified using the same amplification condition; 2.5 µg aliquots of amplified RNAs (aRNAs) from each cancerous tissue and from the control were reverse transcribed in the presence of Cy5-dCTP and Cy3-dCTP, respectively. The relative expression ratio was derived from the value of the expression of CDCA1 mRNA in cancer cells divided by that in normal counterpart (Fig. 1*a*). In Figure 1*b*, the relative expression ratio of normal tissues was derived from the value of the expression of CDCA1 mRNA in each normal tissue divided by the mean value of the expression of CDCA1 mRNA in the mixture of an equal amount of RNA derived from all normal tissues tested.

Northern blot analysis and reverse transcription-PCR

Northern blot analysis was done as described previously.[22] The integrity of RNA in the formalin-Mops gels was checked using electrophoresis. Gels with 20 µg of total RNA per lane were blotted onto a nylon membrane (Hybond N; Amersham, Piscataway, NJ). Poly(A) RNA blots of human tissues were also used. The membranes were hybridized with a CDCA1-specific cDNA probe (1113–1924 bp) labeled with [32P]dCTP. RT-PCR analysis of cancer cell lines and NSCLC tissues was done as described previously.[23] CDCA1 gene-specific PCR primer sequences were as follows: sense, 5′-CCCAGATATAATGTAGCTGAGATT-3′; antisense, 5′-CTCCTGGTGTGCGGCGTTTA-3′, and used RT-PCR reactions consisting of initial denaturation at 94°C for 5 min and 32 amplification cycles at an annealing temperature of 55°C.

Histologic and immunohistochemical analysis

Immunohistochemical examinations of the CDCA1 protein in patients' cancer tissues were also done as described previously.[24,25] The primary antibody used in this study, mouse monoclonal antibody specific to Nuf2 (synonymous to CDCA1), was purchased from Abcam (Cambridge, UK).

Patients, blood samples and cell lines

Blood samples from patients with NSCLC were obtained during routine diagnostic procedures after obtaining informed consent from the patients in Kumamoto University Hospital from September 2006 to December 2007. A TAP-deficient and HLA-A*0201-positive cell line (T2) and the CDCA1^{+} human pancreas cancer cell line (PANC1) were purchased from Riken Cell Bank in Tsukuba, Japan. CDCA1-negative human colon cancer cell line COLO201, CDCA1-positive LC cell lines EBC-1 and LC-1sq were kindly provided by Health Science Research Resources Bank in Tsukuba, Japan. The expression of HLA-A2 on the cell surface was examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 (One Lambda, Canoga Park, CA), to select HLA-A2-positive blood donors and target cell lines for CTL assay.

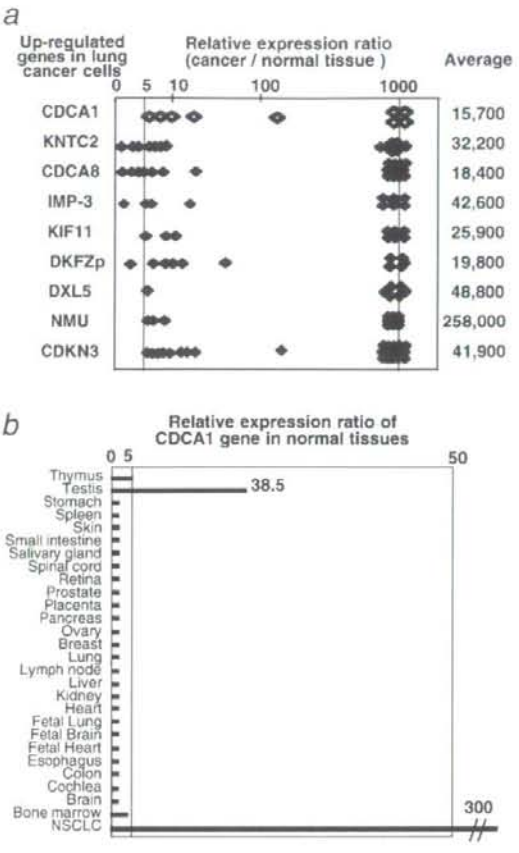

FIGURE 1 – Markedly and frequently enhanced expression of a novel cancer testis antigen, CDCA1, in NSCLC tissues as based on cDNA microarray analysis. (*a*) A list of upregulated genes in NSCLC tissues. These genes were overexpressed in cancer cells when compared with normal counterpart. All 9 patients, who could be investigated by microarray analysis, showed high expression of *CDCA1* in cancer cells. The relative expression ratio was derived from the value of the expression of *CDCA1* mRNA in cancer cells divided by that in normal counterpart. (*b*) The relative ratio (RR) of expression of human *CDCA1* mRNA in 9 NSCLC tissues and disease-free tissues examined by cDNA microarray analysis. *CDCA1* gene was highly expressed only in testis among normal tissues, as based on cDNA microarray analysis. The relative expression ratio of normal tissues was derived from the value of the expression of *CDCA1* mRNA in each normal tissue divided by the mean value of the expression of *CDCA1* mRNA in the mixture of equal amount of RNA isolated from all normal tissues tested.

TABLE I – EXPRESSION OF CDCA1 IN NSCLC AND OTHER CANCEROUS TISSUES[1]

Cancerous tissue	Frequency of tumor overexpressing CDCA1		Average of relative expression ratio
	N	%	
Small cell lung cancer	15/15	100	10,299
Nonsmall cell lung cancer	9/9	100	15,694
Cholangiocellular cancer	12/12	100	6,885
Bladder cancer	28/28	100	3,880
Renal cell cancer	7/7	100	15
Prostate cancer	17/20	85	24,653
CML	14/17	82	27,898
Malignant lymphoma	7/9	78	26,159
Cervical cancer	12/16	75	10,068
Osteosarcoma	14/19	74	88,855
Breast cancer	25/39	64	3,070
Sarcoma	18/36	50	6,134
Colon cancer	4/10	40	2,996
Esophageal cancer	4/18	22	3.4
AML	0/16	0	1.6
Gastric cancer	0/5	0	0

[1]The expression level of CDCA1 also increased in all cancerous tissues of small cell lung cancer, cholangiocellular cancer, bladder cancer and renal cell cancer based on cDNA microarray analysis.[21,28–30]

Lentiviral gene transfer

A lentiviral vector-mediated gene transfer was performed as described.[26] Briefly, 17 μg of CSII-CMV-RfA and CSIIEF-RfA self-inactivating vectors[27] carrying CDCA1 cDNAs and 10 μg of pCMV-VSV-G-RSV-Rev and pCAG-HIVgp were transfected into the 293T cells grown in the 10-cm culture dish using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 60 hr of transfection, the medium was recovered and the viral particles were pelleted by ultracentrifugation (50,000*g*, 2 hr). The pellet was suspended in 50 μl of RPMI 1640 medium and then 10 μl of viral suspension was added to 5×10^4 COLO201 cells per well in a U-bottom 96-well plate. The expression of the transfected *CDCA1* gene was confirmed by Western blot analysis.

Induction of CDCA1-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay

Human CDCA1-derived peptides carrying binding motifs for HLA*0201-encoded molecules were identified using the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and 40 peptides (purity > 90%) carrying HLA-A2 (A*0201) binding motifs were synthesized (Table I).[21,28–30] The immunizations of mice with peptides were done as previously described.[22] Then, 6 days after the start of *in vitro* culture, an enzyme-linked immunospot (ELISPOT) assay was done, according to the manufacturer's recommendations. Mouse IFN-γ ELISPOT kit (BD Biosciences, San Jose, CA) was used for the detection of CDCA1 epitope reactive mouse CTLs secreting IFN-γ in response to the syngenic BM-DC pulsed or unpulsed with each peptides.[31,32]

Induction of CDCA1-reactive human CTLs

Monocyte-derived DCs were used as antigen-presenting cells to induce CTL responses against peptides presented in the context of HLA. The DC were generated by *in vitro* culture as previously described.[33,34] Briefly, PBMC isolated from a healthy volunteers or a NSCLC patient positive for HLA-A*0201 using Ficoll-Plaque (GE Healthcare UK, Buckinghamshire, UK) solution were sorted to the $CD8^+$ population and the $CD14^+$ population with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate DCs, the $CD14^+$ population was cultured in the presence of 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; provided by PeproTec, NJ) and 10 ng/ml interleukin (IL)-4 (PeproTec) in AIM-V (Invitrogen) containing 2% heat-inactivated autologous plasma. After 4 days of culture, OK-432 was added into the dish to make the DCs mature. After 5 days, we started to culture the cytokine-generated DCs, and they were pulsed with 20 μg/ml HLA-A2-binding peptides in the presence of 4 μg/ml β2-microglobulin (Sigma-Aldrich, St. Louis, MO) for 2 hr at 37°C in AIM-V. These peptide-pulsed DCs were then irradiated (3,500 cGy) and mixed at a 1:50 ratio with autologous $CD8^+$ T cells, obtained by positive selection of PBMCs with anti-CD8 microbeads (Miltenyi Biotec). These cultures were set up in 48-well plates; each well contained 1×10^4 peptide-pulsed DCs, 5×10^5 $CD8^+$ T cells and 10 ng/ml human IL-7 (Wako, Osaka, Japan) in 0.5 ml AIM-V with 2% autologous plasma. After 3 days, these cultures were supplemented with human IL-2 (PeproTec) to a final concentration of 20 IU/ml. On days 12 and 19, the T cells were further restimulated with the peptide-pulsed autologous DCs. We prepared the DCs each time in the same way as described earlier. The antigen-specific responses of the CTLs were investigated using chromium release assay and IFNγ ELISPOT assay on 6 days after the third round of peptide stimulation was performed on day 25.

HLA-A*0201 tetramers labeled with PE and bound by the $CDCA1_{351-359}$ peptide was obtained from Medical and Biological Laboratories, Nagoya, Japan.

CTL responses against target cells

The CTLs were cocultured with each cancer cell line or peptide-pulsed/unpulsed T2 cells as a target cell at the indicated effector/target ratio, and a ^{51}Cr release assay and IFN-γ ELISPOT assay were done as described.[32,35,36] Briefly, the target cells were labeled with 3.7 KBq $Na_2{}^{51}Cr_4$ (Perkin Elmer Life Sciences, Waltham, MA) for 1 hr at 37°C in a CO_2 incubator. The labeled target cells were rinsed 3 times, and the peptide-pulsed target cells were prepared by incubating the cells with 20 μg/ml peptide for 3 hr at 37°C. The target cells were mixed with the effector cells in a final volume of 200 μl in flat-bottomed microtiter plates and incubated. After 6 hr incubation, 50 μl of the supernatant was collected from each well and the radioactivity was quantified using a gamma counter. The specific cytotoxicity was evaluated by calculating the percentage of specific ^{51}Cr release as described.[37] An ELISPOT assay was carried out as described previously.[32]

Results

Markedly enhanced expression of CDCA1 mRNA in NSCLC and other cancerous tissues and cell lines

Using a cDNA microarray representing 27,648 genes, we chose 9 genes which were overexpressed in the great majority of NSCLC tissues among 37 cases of NSCLC in comparison to their adjacent normal counterpart. The relative expression ratios (cancer/normal counterpart) of all 9 genes were more than 15,000 (Fig. 1*a*). Thereafter, we analyzed the expression of these genes using a cDNA microarray analysis in 28 kinds of normal tissues (including 4 embryonic tissues) (Fig. 1*b*). The relative expression ratio of CDCA1 was more than 15,000 (mean: 15,694; range: 6.5–54,778) in cancerous tissues isolated from 9 patients with NSCLC available from the cDNA microarray data (Fig. 1*a*). In addition, the *CDCA1* gene was strongly expressed only in the testis among 24 adult normal tissues, as based on cDNA microarray analysis (Fig. 1*b*). Consequently, we identified CDCA1 to be an ideal target for immunotherapy of NSCLC patients.

Moreover, to confirm the results obtained from the cDNA microarray analyses, we examined the expression of the *CDCA1* gene in NSCLC cell lines and normal tissues at the mRNA level by using RT-PCR and Northern blot analysis. The *CDCA1* gene was significantly expressed only in the testis among adult normal tissues in both the RT-PCR and Northern blot analyses (Figs. 2*a* and 2*b*), in accordance with the results of the cDNA microarray analysis. The LC cell lines including 1–89, A549, PC14, RERF-LC-KO (adenocarcinoma: ADC), EBC-1, LC-1sq (squamous cell