特集 ○ 癌ワクチン—最近の進歩 ○

癌胎児性抗原 Glypican-3 を標的とした癌ワクチン療法

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要旨　われわれは，Glypican-3（GPC3）が肝細胞癌（HCC）に高発現する新規癌胎児性抗原であり，腫瘍マーカーとして有用であること，またBALB/cマウスにGPC3由来のペプチドを負荷した樹状細胞を免疫すると，GPC3発現腫瘍の増殖が抑制されることを報告している。今回，ヒトCTLが認識するGPC3由来のHLA-A2あるいはHLA-A24拘束性エピトープペプチドを同定し，これらを用いてHCC患者の末梢血単核球（PBMC）からGPC3ペプチド特異的細胞傷害性Tリンパ球（CTL）が誘導できるか否かを検討した。HLA-A2陽性GPC3陽性HCC患者のPBMCよりGPC3$_{144\text{-}152}$ペプチドを用いて，8名中5名から，またHLA-A24陽性GPC3陽性HCC患者のPBMCよりGPC3$_{298\text{-}306}$ペプチドを用いて6名中4名から，各CTLエピトープに特異的なCTLを誘導できた。また，これらのCTLはNOD/SCIDマウスに移植した，GPC3を発現するヒトHCC細胞株の増殖を抑制した。これらの結果から，GPC3由来のペプチドは，HCC患者の免疫療法の新たなターゲットとして，その臨床応用が期待できる。

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Identification of CTL Epitopes of Glypican-3 Useful for Cancer Immunotherapy

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Summary

We previously reported that Glypican-3 (GPC3) was overexpressed specifically in hepatocellular carcinoma (HCC) in humans, and it was useful as a novel tumor marker for HCC. We also reported that the pre-immunization of BALB/c mice with bone-marrow-derived dendritic cells pulsed with the H-2K^d-restricted mouse GPC3$_{298\text{-}306}$ (EYILSLEEL) peptide prevented the growth of tumor expressing mouse GPC3.

Because of similarities in the binding peptide motifs between H-2K^d and HLA-A24 (A*2402), the GPC3$_{298\text{-}306}$ peptide thus seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC. Therefore, we investigated whether or not the GPC3$_{298\text{-}306}$ peptide could induce GPC3-specific CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A24 (A*2402)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice (Tgm) to identify the HLA-A2 (A*0201)-restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients. We found that the GPC3$_{144\text{-}152}$ (FVGEFFTDV) peptide could induce peptide-specific CTLs in HLA-A2.1 (HHD) Tgm without inducing autoimmunity. In 5 out of 8 HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3$_{144\text{-}152}$ peptide-

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specific CTLs were generated from PBMCs by *in vitro* stimulation with the peptide. The $GPC3_{298-306}$ peptide-specific CTLs were also generated from PBMCs in 4 of 6 $HLA-A24^+$ $GPC3^+$ HCC patients, and the inoculated CTLs could attack the human HCC tumor mass implanted into NOD/SCID mice.
Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Key words : Cancer immunotherapy, CTL, HCC, Glypican-3 (GPC3)
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はじめに

肝細胞癌（HCC）の罹患数は，欧米およびアジア諸国において依然として増大している。HCCは治療後も高頻度に再発を繰り返すため予後不良な癌であり，肝炎，肝硬変から発生したごく初期の癌に対する早期治療法や治療後の再発予防のために有効な補助療法の確立が望まれる。Glypican-3（GPC3）はHCCに高発現し，腫瘍免疫のターゲットとして理想的な癌胎児性抗原である。われわれは，HCCに対する免疫療法の新たなターゲットとしてGPC3に着目し，その有用性に関して前臨床試験を終了し，臨床試験を開始する予定である[1-3]。

I．HCCに対する免疫療法

慢性肝炎，肝硬変患者におけるHCCの発症予防やHCC術後における術後化学療法は，未だ開発途上にある。HCCに対する免疫療法についても，1990年代よりlymphokine activated killer（LAK）cells, tumor infiltrating lymphocytes（TIL），peripheral blood mononuclear cell（PBMC）を用いた養子免疫療法，DCワクチン療法，AFP由来ペプチドワクチン療法など試みられているが，未だ標準的な治療法として確立されていない[4]。HCCにおいて高発現する癌特異的抗原も多数報告されており，各施設でその有用性が検討されている[4]（表1，2）。

II．新規癌胎児性抗原（GPC3）

われわれは，東京大学医科学研究所ヒトゲノム解析センターの中村祐輔博士らとの共同研究により，癌部と非癌部のcDNAマイクロアレイ解析を用いてHCC特異的に高発現する遺伝子としてGPC3を同定した（図1）。

1．GPC3の構造と機能

膜結合型のGlypicanファミリーは，現在までのところ6種類が報告されている[5]。GPC3は，580アミノ酸からなる60 kDのコア蛋白質にヘパラン硫酸プロテオグリカンの糖鎖修飾が加わった膜蛋白質で，C末端がGPIアンカーにより形質膜に結合している。Piliaらは，X染色体（Xq26）連鎖疾患である巨人症の一種Simpson-Golabi-Behmel症候群（SGBs）において，GPC3の遺伝子変異を報告している。また，GPC3ノックアウトマウスでも，SGBsと同様に巨大化などの表現型を示すことがわかっている。GPC3の機能としては，ある種の腫瘍細胞では増殖を抑制したり，あるいはアポトーシスに関連があると報告されている[6]。近年，GPC3コア蛋白質が直接Wntと結合することによりWnt signalを活性化し，HCCの増殖を促進することが報告されている[7]。

2．HCC組織におけるGPC3の発現

われわれは，遺伝子の発現量の差が，その遺伝子産物である蛋白質量の差として反映されているか否かをRT-PCR法ならびに組織切片における免疫染色法を用いて確認した（図2）。肝臓組織は，胎児期においてGPC3を発現するが出生後発現しなくなり，HCCにおいては再び発現するためGPC3は癌胎児性蛋白質としての性格を有しており，恐らく胎児の発生に重要な役割を担っていると推測される。一般に，癌胎児性蛋白質は腫瘍の進行において重要な役割を担っているとは考えられていないが，腫瘍マーカーまたは免疫療法の標的として使用されてきた。

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表1 HCCに高発現する癌抗原に関する過去の報告のまとめ*
(Butterfield, L.H.: *Gastroenterology*, 2004. を基に情報を追加)

Study (Year)	GPC-3	MAGE-1	MAGE-2	MAGE-3	MAGE-4	MAGE-10	MAGE-12	SSX-1	NY-ESO1
Yamashita (1996)		80							
Kariyama (1999)		78		42					
Tahara (1999)		68	30	68		30	30		
Chen (2001)								80	
Mou (2002)		70		53					
Luo (2002)		19		24	4			38	0
Chen (2003)		66		70	20	36			40
Nakatsura (2003)	80								
Korangy (2004)									24

*：HCC組識における各腫瘍抗原mRNAの発現頻度（%）

表2 HCC対するワクチン療法の臨床試験に関する過去の報告のまとめ（文献[4]より）

Strategy	Author (Year)	Patients	Setting	Responses
Dendritic cells (DC vaccine)	Ladhams, *et al* (2002)	2 metastatic	GM/IL-4 DC + tumor	1 patient slowed tumor growth
	Iwashita, *et al* (2003)	10 unresectable	GM/IL-4 DC + tumor lysate + TNF + KLH	1/10 MR
	Stift, *et al* (2003)	2 HCC of 20 total	GM/IL-4 DC + tumor lysate + TNF + IL-2	No PR or CR
AFP peptidse	Butterfield, *et al* (2003)	6 stage Ⅳa and Ⅳb	AFP peptidse in Montanide adiuvant	No PR or CR

ワクチンを基盤とした免疫療法に関する臨床試験のみを抜粋した。
GM：GM-CSF, KLH：keyhole limpet hemocyanin, AFP：α-fetoprotain, MR：minor response,
PR：partial response, CR：complete response

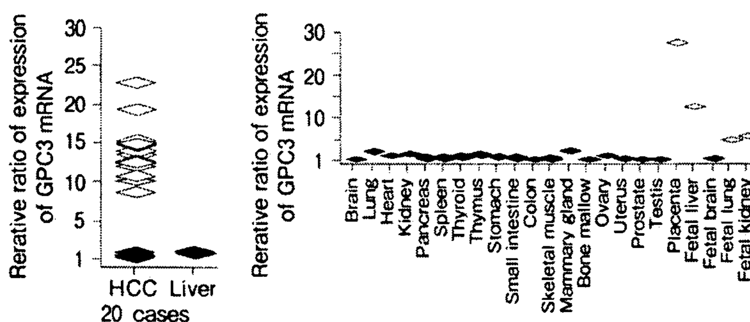

図1 HCC 20例の癌部，非癌部および多様な正常臓器におけるGPC3遺伝子発現のcDNAマイクロアレイ解析データ（東京大学医科学研究所ヒトゲノム解析センター：中村祐輔博士らの研究成果，Okabe, H., *et al.*: *Cancer Res.* 61: 2129, 2001.）
HCC患者20例の癌部と非癌部における23,040種類の遺伝子の発現を比較検討し，発現の比が5以上の遺伝子を16種類選んだ。さらに胎生期の4臓器を含む23臓器の正常組織において，各遺伝子の発現プロファイルを解析して，胎生期の組織あるいは免疫学的に隔離された胎盤や精巣にしか発現しない遺伝子GPC3を探しだした。GPC3は，HCC患者20例中16例で癌部/非癌部の発現の比が5以上（平均396.2）で，胎盤や胎生肝，胎生腎に発現する以外はほとんどの成人正常臓器に発現を認めない癌胎児性抗原をコードする遺伝子であった。

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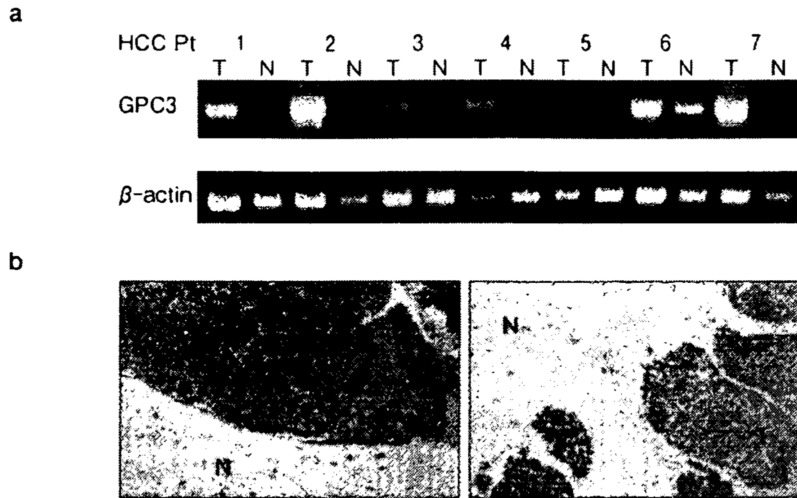

図2 HCC組織におけるGPC3の発現

a：HCC組織の癌部（T），非癌部（N）におけるGPC3mRNAの発現の有無をRT-PCR法にて検討し，癌部においてのみGPC3の発現を認めた。

b：HCC組織切片におけるGPC3蛋白質の発現を，抗GPC3抗体を用いた免疫組織学的解析により確認した。

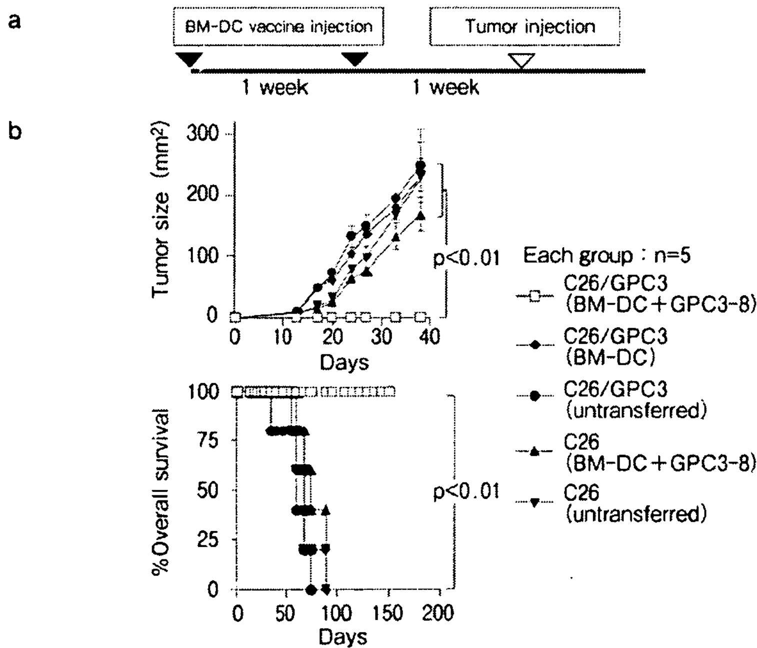

図3 マウスにおけるGPC3ペプチドを負荷したBM-DCワクチンによる腫瘍抑制効果

a：実験のプロトコール。BM-DCは1週間ごとに2回，BALB/cマウスの腹腔内に5×10^5個を投与した。その1週間後に腫瘍を3×10^4個背部の皮下に移植した。

b：抗腫瘍効果の検討。GPC3-8ペプチドを負荷したBM-DCワクチンを投与した後に，同系マウス由来の大腸癌細胞株C26に，マウスGPC3遺伝子を強制発現させた細胞株（C26/GPC3）を皮下移植した群では腫瘍の拒絶が認められた（上段）。さらに同群マウスにおいて，生存期間の著明な延長が観察された（下段）。

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表3 HLA-A2あるいはHLA-A24陽性HCC患者（それぞれPt-A2, Pt-A24）の約50%において，GPC3特異的なCTLが誘導された

Patients	Age	Gender	State of tumor†	GPC3 expression#	HKA expression★	CTL induction*
Pt-A2-1	80	F	Ⅲa	+	+	+
Pt-A2-2	72	M	Ⅱ	+	+	+
Pt-A2-3	67	F	Ⅱ	ND	ND	+
Pt-A2-4	54	M	Ⅰ	+	+	+
Pt-A2-5	57	M	Ⅰ	ND	ND	−
Pt-A2-6	66	M	Ⅰ	−	−	−
Pt-A2-7	54	M	Ⅲa	+	+	−
Pt-A2-8	73	M	Ⅱ	ND	ND	+
Pt-A2-9	68	F	Ⅲa	+	+	−
Pt-A2-10	54	M	Ⅱ	+	+	−
Pt-A24-1	60	M	Ⅳa	+	+	+
Pt-A24-2	57	M	Ⅳa	+	+	−
Pt-A24-3	75	F	Ⅲa	+	+	+
Pt-A24-4	59	M	Ⅲa	ND	ND	+
Pt-A24-5	52	M	Ⅳb	−	+	−
Pt-A24-6	65	M	Ⅰ	ND	ND	+
Pt-A24-7	61	M	Ⅰ	ND	ND	+
Pt-A24-8	74	M	Ⅱ	ND	ND	−
Pt-A24-9	59	M	Ⅳb	−	−	−
Pt-A24-10	69	M	Ⅳa	+	+	−
Pt-A24-11	72	M	Ⅱ	−	+	−
Pt-A24-12	61	M	Ⅲa	+	+	+

†：TNM分類を用いた，#：免疫染色を用いて腫瘍周囲組織と比較した，★：免疫染色にて膜が染色された場合を陽性とした，*：GPC3発現HCC細胞株HepG2に対する細胞傷害活性が，E/T比20で20%以上観察された場合にCTLを誘導できたと判断した。

3. 腫瘍マーカーとしてのGPC3の有用性

われわれは，ELISA法を用いてHCC患者の約40%の血清中のGPC3蛋白について検討したところ，HCC患者の血清中にのみ検出されるが，健常人，その他の良性肝疾患患者や他の癌種の患者ではまったく検出されなかった。さらにHCC切除手術後に血清GPC3は著明に減少したことから，GPC3がHCCの腫瘍マーカーとして有用であることを報告した[8]。HCCの腫瘍マーカーとしてのGPC3の有用性に関しては，他施設からも同時期に報告されているが[9,10]，早期診断や治療効果の判定などの臨床応用が期待される。

Ⅲ．抗腫瘍免疫療法のターゲットとしてのGPC3の有用性

1. マウスにおける腫瘍免疫の解析

発現の組織特異性が優れていることから，Nakatsuraらは，この新規癌胎児性抗原GPC3が理想的な腫瘍拒絶抗原になり得るかどうかを検討した。日本人の約60%が所有するHLA-A24とBALB/cマウスのclass Ⅰ分子のK^dに結合するペプチドの構造モチーフは，非常に一致していることがわかっている。さらに，ヒトとマウスのGPC3では，アミノ酸配列のレベルで95%以上のホモロジーを認めることから，ヒトとマウスのGPC3でアミノ酸配列が完全に一致し，HLA-A24，K^dのいずれにも結合し得るGPC3由来のペプチドを合成した。これらをBALB/cマウスに免疫して解析し，K^d分子に結合して細胞傷害性Tリンパ球（CTL）に提示される（K^d拘束性）

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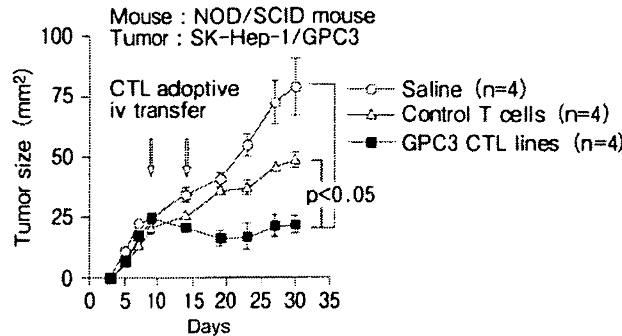

図4 GPC3発現ヒトHCC細胞株に対する養子免疫療法の有効性
免疫不全マウスであるNOD/SCIDマウスに移植したGPC3発現ヒトHCC細胞株に対する，ヒトGPC3特異的CTLの養子免疫による抗腫瘍効果を検討した。GPC3エピトープペプチドで誘導したヒトCTLを投与すると，コントロール群に比べ有意に腫瘍の増大が抑制された。NOD/SCIDマウスの背部の皮下に，ヒトHCC細胞株SK-Hep-1にGPC3遺伝子を強制発現させたSK-Hep-1/GPC3を1×10^7個移植し，5×5 mmの大きさになったところでCTLを8×10^7個iv投与した。HCC患者のPBMCをGPC3エピトープペプチドで刺激して誘導したCTL投与群（■）と，コントロールとしてHIVエピトープペプチドで誘導したCTL投与群（△），生理食塩水のみを投与した群（○）の間で比較すると，GPC3特異的CTL投与群ではコントロール群に比べ，有意に腫瘍増殖が抑制された。

CTLエピトープペプチドを同定した[1]。このエピトープペプチドを負荷した骨髄由来の樹状細胞（BM-DC）ワクチンをBALB/cマウスの腹腔内に予防的に投与した場合，コントロール群に比べ，GPC3発現マウス大腸癌腫瘍の増殖は著明に抑制された（図3)。このエピトープペプチドはHLA-A24によっても提示され，ヒトでも同様にCTLエピトープとなる可能性があると思われた。またMotomuraらは，マウスGPC3を遺伝子導入したマウスES細胞より分化誘導した樹状細胞（ES-DC-GPC3）を樹立した。ES-DC-GPC3をマウスに免疫することにより，*in vivo*においてGPC3特異的なCTLが誘導され，移植されたGPC3発現腫瘍の増殖転移が抑制されることを報告している[2]。

2. HCC患者における腫瘍免疫の解析

日本人のHLA class I対立遺伝子のうち，HLA-A24（A*2402）は日本人の約60％が所有し，HLA-A2（A*0201）は約30％が所有する，ありふれた対立遺伝子である。そこで，ヒトとマウスのGPC3に保存されたアミノ酸配列をもつペプチドで，HLA-A2（A*0201）に結合すると推定されるGPC3由来の9～10個のアミノ酸からなるペプチドを9種類選択した。このうち，HLA-A2トランスジェニックマウス（HLA-A2 Tgm）に最も強く，GPC3特異的なCTLを誘導できるエピトープペプチドをELISPOTアッセイにて検討した結果，ペプチドA2-3：$GPC3_{144-152}$がCTLエピトープ候補として同定された。このGPC3 A2-3ペプチドを負荷したBM-DCを2回免疫したHLA-A2 Tgmでは，重要臓器（脳，皮膚，心，肺，肝，腎）への自己免疫反応は生じておらず，その安全性が示唆された。

HLA-A2拘束性CTLエピトープペプチド$GPC3_{144-152}$と，HLA-A24拘束性CTLエピトープペプチド$GPC3_{298-306}$を用いて，HLA-A2またはHLA-A24陽性のHCC患者のPBMCから，ペプチド特異的CTLの誘導を試みた。その結果，それぞれのペプチドについてペプチド特異的にCTLが誘導され，HLA-A2陽性GPC3陽性HCC患者のPBMCより$GPC3_{144-152}$ペプチドを用いて8名中5名から，またHLA-A24陽性GPC3陽性HCC患者のPBMCより$GPC3_{298-306}$ペプチドを用いて6名中4名から，各CTLエピトープ特異的なCTLを誘導できた（表3)。

さらに，NOD/SCIDマウスにGPC3遺伝子を

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強制発現させたヒトHCC細胞株SK-Hep1/GPC3を皮下注射して生着させた後に，HLA-A2拘束性エピトープペプチドGPC3$_{144-152}$あるいはHLA-A24拘束性エピトープペプチドGPC3$_{298-306}$で刺激することにより，HCC患者のPBMCより誘導されたヒトCTL株を養子免疫した。GPC3エピトープペプチドにて誘導したCTL株を静脈内投与したNOD/SCIDマウスでは，コントロールのT細胞株あるいは生理食塩水のみを投与した群と比較して，有意差をもって腫瘍の増殖抑制が観察された（図4)。現在，国立がんセンター東病院にてHCC患者を対象にして，これらのペプチドを用いた癌免疫療法の臨床試験を計画中である。

おわりに

GPC3由来のCTLエピトープは，HCCの免疫療法の新たなターゲットとして，その臨床応用が期待される。腫瘍の免疫逃避に対抗するためには多様な腫瘍拒絶抗原のレパートリーを確立することが望まれる。GPC3がその一つとして，これを用いた免疫療法がHCCの再発，発症防止に寄与することを期待したい。

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Heat shock protein 105 is overexpressed in squamous cell carcinoma and extramammary Paget disease but not in basal cell carcinoma

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basal cell carcinoma, extramammary Paget disease, heat shock protein 105, squamous cell carcinoma

Conflicts of interest
None declared.

Summary

Background Heat shock protein (HSP) 105 is a 105-kDa protein, recently discovered by serological analysis of recombinant cDNA expression libraries prepared from tumour cells (SEREX), and is still undergoing intensive research. SEREX can define strongly immunogenic tumour antigens that elicit both cellular and humoral immunity. Previous studies have shown that HSP105 is a cancer testis antigen and is overexpressed in various internal malignancies. The expression of HSP105 has not been studied in skin cancers.

Objectives To assess the expression of HSP105 in skin cancers including extramammary Paget disease (EMPD), cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC).

Methods Samples of EMPD (n = 25), SCC (n = 23, of which three were metastatic lesions) and BCC (n = 23) were collected from patients treated in our department between January 2002 and December 2004. Western blot and immunohistochemical staining methods were used to investigate the expression of HSP105.

Results Results of Western blot analysis showed overexpression of HSP105 in EMPD and SCC, and minimal expression in BCC. Immunohistochemistry results showed that 56% of EMPD, 60% of primary and 100% of metastatic SCC highly expressed HSP105 while only 13% of BCC lesions showed increased staining.

Conclusions EMPD and SCC overexpress HSP105 while BCC does not. Tumours overexpressing HSP105 present ideal candidates for vaccination by HSP105-derived peptides or DNA.

Heat shock protein (HSP) 105 is a mammalian stress protein that belongs to the HSP110 family.[1] It is released by tissues in response to a wide variety of stresses including infection, ischaemia, heat stress and tumours.[2,3] Studies have shown that the molecule is involved as a biochemical mediator of heat-induced apoptosis by binding to p53 at scrotal temperatures and dissociating from it at suprascrotal temperatures in testicular germ cells.[4] HSP105 consists of α and β components. The α-component is 105 kDa and the β-component is a truncated form, 90 kDa in size, and is specifically induced by heat stress at 42 °C.[5,6] It is important to investigate and isolate tumours which overexpress HSP105 in order to target them for immunotherapy. Studies on colorectal carcinoma and melanoma cell lines in mouse hosts showed that HSP105 DNA vaccination could stimulate HSP105-specific tumour immunity leading to tumour regression.[7]

Kai et al.[8] showed that HSP105 is overexpressed in a variety of intra-abdominal carcinomas such as oesophageal squamous cell carcinoma (SCC), colon and pancreatic adenocarcinoma, and others. No studies have been done to investigate the expression of HSP105 in cutaneous malignancies. We therefore aimed to evaluate the expression of HSP105 in extramammary Paget disease (EMPD), SCC and basal cell carcinoma (BCC). This is the first study to investigate the expression of HSP105 in human skin cancers.

Materials and methods

Tissue specimens

Tissue samples for Western blot analysis were frozen in liquid nitrogen soon after excision and kept at −80 °C. The specimens consisted of one of EMPD, two of SCC, one of BCC and two of normal skin.

Archival formalin-fixed, paraffin-embedded tissues were used for immunohistochemical analysis; the clinical characteristics of

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the patients are as follows. Twenty-five specimens of EMPD were obtained from 10 women and 15 men, age range 51–87 years (mean 74). Twenty-three specimens of SCC, of which three were metastatic lymph nodes, were analysed. The SCC population comprised 13 women and 10 men, age range 55–95 years (mean 78·9). Nine women and 14 men with a diagnosis of BCC had age range 51–83 years (mean 70·6).

The specimens were obtained from patients who underwent surgery between January 2002 and December 2004 in the Departments of Dermatology and Plastic and Reconstructive Surgery at Kumamoto University Hospital, from whom informed consent was obtained before surgery. Haematoxylin and eosin-stained sections were used for the initial light microscopy assessment.

Western blot

The frozen tissues were homogenized and 20 μL of the supernatant subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis before transfer to a nitrocellulose membrane. Blocking was achieved by incubating the membrane in 5% skimmed milk/Tris-buffered saline–Tween 20 (TBST) overnight at 4 °C. HSP105 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used at a concentration of 1 : 500 and incubated with the membrane for 90 min at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated antirabbit antibody (1 : 3000) for 30 min. Membranes were thoroughly washed with 1 mol L^{-1} NaCl/TBST. Signals were detected using the ECL (enhanced chemiluminescence) system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Immunohistochemistry

Paraffin sections (4 μm) were used for immunohistochemical examination after deparaffinization in xylene and rehydration through graded alcohols. Antigen retrieval was achieved by microwave treatment for 10 min in citric acid (0·01 mmol L^{-1}, pH 6·0), followed by cooling at room temperature for 60 min. Nonspecific staining was blocked with 5% normal horse serum. HSP105 antibody (Santa Cruz Biotechnology) was used at a concentration of 1 : 100 and incubated with the specimens overnight in a humidifying chamber. Endogenous peroxidase was blocked by immersing the sections into 1·5% hydrogen peroxide diluted with methanol for 1 min. Using the Envision method, antirabbit polyclonal antibody (EnVision+; DakoCytomation, Carpinteria, CA, U.S.A.) was used neat and incubated for 60 min at room temperature. Colour signalling was done using the 3, 3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan)-based detection method. Haematoxylin was used as a counterstain.

Immunohistochemical evaluation incorporated the percentage of stained cells per specimen and the staining intensity, compared with that of adjacent normal tissues as an internal control. The histochemical score (HSCORE)[9] was used to standardize the results of all three malignancies to allow for comparison. The HSCORE was calculated as follows: HSCORE = P_i (i + 1), where i = 0, 1, 2 and P_i (estimated percentage of stained tumour cells) varies from 0 to 100%. Staining intensity of tumour cells was graded according to the following three grades: 0, weak/negative; 1, distinct staining; 2 strong staining. A mean HSCORE of 100 was considered as overexpression of HSP105.

Statistical analysis

HSP105 expression was analysed using the nonparametric Mann–Whitney test, StatView version 5.0 for Windows (SAS Institute, Cary, NC, U.S.A.). $P < 0·05$ was considered significant.

Results

Western blot

Western blot analysis showed overexpression of HSP105 in EMPD and SCC, and low expression in BCC and normal skin (Fig. 1). HSP105 is constitutively expressed by normal skin in small amounts as evidenced by a visible but light band of HSP105.

Immunohistochemistry

Two investigators (F.C.M., T.K.) viewed all tissue sections. Figure 2 shows the staining pattern of EMPD, SCC and BCC.

EMPD showed positive staining of both the nucleus and cytoplasm, with a heterogeneous staining intensity; however, the sialomucin (mucous) component of the large Paget cells stained negative. Adjacent normal apocrine and eccrine glands were negative. The heterogeneity of staining necessitated the use of the HSCORE, which incorporates both intensity and distribution of staining, for data analysis. The HSCORE for

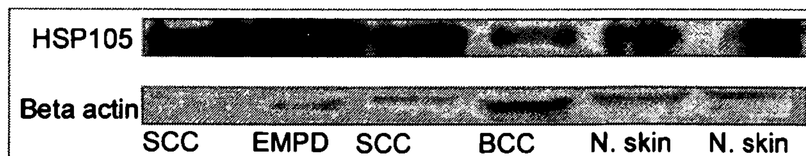

Fig 1. Western blot analysis of heat shock protein (HSP) 105 expression in squamous cell carcinoma (SCC), extramammary Paget disease (EMPD), basal cell carcinoma (BCC) and normal skin. The β-actin band was used as a control.

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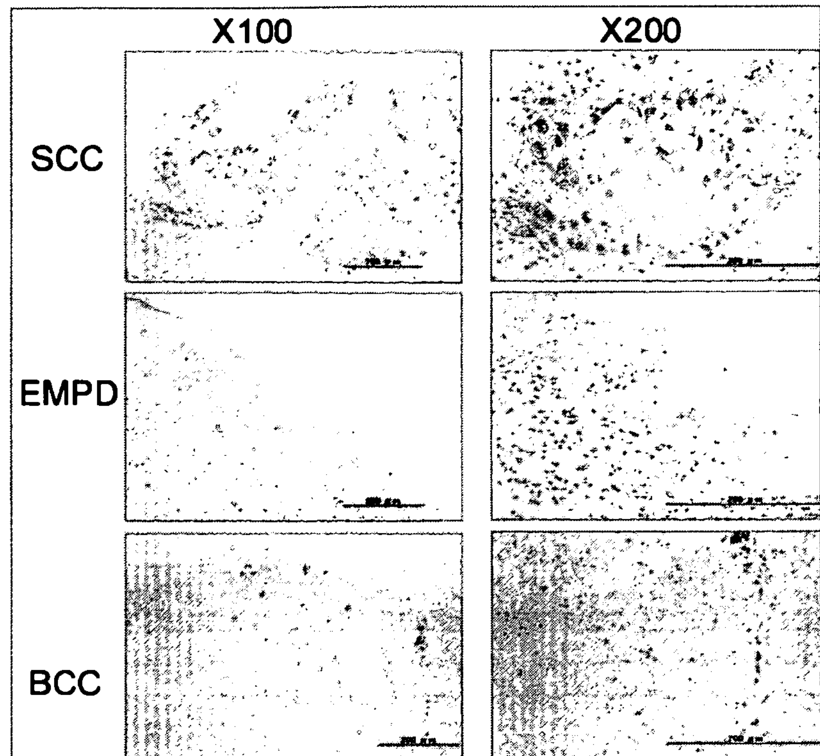

Fig 2. Immunohistochemical staining of heat shock protein (HSP) 105 in squamous cell carcinoma (SCC), extramammary Paget disease (EMPD) and basal cell carcinoma (BCC). The photomicrographs show low and high power views, respectively. SCC and EMPD highly express HSP105 while BCC is negative. Scale bar = 200 μm.

EMPD ranged from 5 to 300 with a mean of 103. Invasive EMPD, with dermal invasion of Paget cells, showed a high-level expression of HSP105 compared with intraepidermal lesions.

SCC highly expressed HSP105 with very strong nuclear and cytoplasmic staining. The HSCORE for primary SCC ranged from 5 to 300 (mean 132). The mean HSCORE for metastatic SCC was 270, indicating a very high level of expression of HSP105.

BCC failed to express HSP105. Even though the overlying epidermis minimally expressed HSP105, the tumour nests exhibited negative expression of the protein (Fig. 2). The HSCORE for BCC ranged from 5 to 60 (mean 17).

Immunohistochemistry results showed that 56% of EMPD, 60% of primary and 100% of metastatic SCC highly expressed HSP105 while only 13% of BCC lesions showed increased staining.

We also investigated by immunohistochemistry the expression of HSP105 in sun-damaged lesions of actinic keratosis; results showed that most specimens exhibited increased staining to HSP105 (data not shown).

Discussion

Although surgical excision is the most common method for treatment of EMPD, SCC and BCC, dermatologists often meet challenging cases. EMPD is an uncommon malignancy arising within the epidermis from native stem cells that differentiate towards glandular cells.[10] The common sites for EMPD are mainly the vulval, penile, scrotal and perianal regions. Often the lesion is so extensive that radical excisions sometimes result in a structural and functional compromise of the adjacent vital structures. Up to 60% of the cases recur[11] and often the tumour extends 2–5 cm beyond that visible under light microscopy; this necessitates repeated excisions, further compromising the integrity of the local structures. We have shown in this study that EMPD and SCC, especially the metastatic lesions, overexpress HSP105, and these tumours present ideal candidates for HSP105-based immunotherapy.

HSPs are present in cells at normal temperatures and act as molecular chaperones, assisting in the folding, unfolding and transport of various proteins. HSP105 is localized in the cytoplasm and nucleus of both stressed and nonstressed cells and it is believed to be translocated from the cytoplasm into the nucleus after heat stress.[12]

In this study we demonstrated by immunohistochemistry and Western blot analysis that HSP105 is overexpressed in EMPD and SCC but not in BCC. Some investigators have implicated HSP105 to be antiapoptotic by binding to p53, the antitumour gene product.[4] This study supports the hypothesis that BCC is a stable tumour as it failed to express the HSP105 protein. Some reports have shown that BCC is a relatively benign and slowly progressing tumour, rarely, if ever, metastasizing. It expresses HSP27, a marker of differentiation and proliferation, expressed in keratinocytes, which helps the cell in repair processes.[13] On the other hand, HSP27 is not expressed by SCC, showing the relative aggressiveness of SCC.[14]

Our study has shown that even though surrounding normal tissues in BCC show minimal expression of HSP105, the BCC nests themselves are negative. Kai *et al.* reported negative

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staining of the adjacent normal epithelium in the colorectal region;[8] we have found that normal epidermis of skin was slightly reactive to HSP105. However, in grading positivity, a score of 1 was allocated to tumour cells whose staining was more intense than the adjacent normal epidermis.

The skin is the basis of protection from environmental factors and sunlight is a major contributor to skin damage. Sunlight is noted to be an aetiological feature in both SCC and BCC. In our patients, BCC was found mainly on the face. The adjacent epidermis of BCC sections, as noted above, stained slightly more than the tumour nests; we therefore queried whether or not sunburn may have a significant role to play in the expression of HSP105 in photodamaged lesions. Actinic keratosis is a premalignant condition to SCC; if left untreated 2–5% of these cases progress to SCC.[15] Cells that highly express HSP105 have an increased thermotolerance; in malignant cells this phenomenon increases the stability of abnormal cells, leading to progression of the diseases.

The detection of HSP105 by serological analysis of recombinant cDNA expression libraries prepared from tumour cells (SEREX) is of great importance as this method detects tumour antigens that elicit strong humoral and cellular immunity.[16] The task is now for immunologists to engineer an HSP105-based vaccine for testing on tumours that have proved to overexpress it. Development of a specific immunotherapeutic agent that can identify microscopic satellite tumours would be a great leap in the management of EMPD. Tamura *et al.*[17] showed that immunotherapy of mice with pre-existing cancers with HSP preparations derived from autologous cancer cells resulted in retarded progression of the primary cancer, reduced metastatic load and prolongation of life span. The effectiveness of anti-HSP90 drugs in the treatment of paediatric neuroblastoma and osteosarcoma was recently tested. It was found that there was a significant reduction in cell survival after exposure to the drugs, alone or in combination with others.[18] HSP105, by virtue of being discovered by SEREX, is highly immunogenic. Recent studies by Miyazaki *et al.*[7] successfully demonstrated that HSP105 DNA vaccination of mice which were challenged with colorectal and melanoma cell lines could elicit tumour rejection through activation of both cellular and humoral immunity. Therefore, advanced or metastatic EMPD and SCC could be good targets for HSP105 immunotherapy. HSP105 DNA vaccination studies for SCC in mice are under way in our laboratory.

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Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

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Abstract **Purpose and Experimental Design:** We previously reported that glypican-3 (GPC3) was overexpressed, specifically in hepatocellular carcinoma (HCC) and melanoma in humans, and it was useful as a novel tumor marker. We also reported that the preimmunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse $GPC3_{298-306}$ (EYILSLEEL) peptide prevented the growth of tumor-expressing mouse GPC3. Because of similarities in the peptide binding motifs between H-2K^d and HLA-A24 (*A*2402*), the $GPC3_{298-306}$ peptide therefore seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. In this report, we investigated whether the $GPC3_{298-306}$ peptide could induce GPC3-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A24 (*A*2402*)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice to identify the HLA-A2 (*A*0201*)–restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients.

Results: We found that the $GPC3_{144-152}$ (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) transgenic mice without inducing autoimmunity. In five out of eight HLA-A2$^+$ GPC3$^+$ HCC patients, the $GPC3_{144-152}$ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the $GPC3_{298-306}$ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusion: Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Hepatocellular carcinoma (HCC) is now spreading rapidly, especially in Asian and Western countries. It is clear that patients with hepatitis B or C–based liver cirrhosis are at high risk for developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at high risk for recurrence (2). Furthermore, the liver function of these patients is often very poor, so further treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence, i.e., adjuvant therapy, is urgently needed. As for melanoma, the age-adjusted incidence rates have been increasing in most fair-skinned populations in recent decades (3). In 2005, it is estimated that 59,580 Americans will be diagnosed to have melanoma, and 7,770 will die from the disease (4).

We and others previously reported that glypican-3 (GPC3) was overexpressed in most types of HCC (5–9) and melanoma in humans (8), and we also previously reported that an H-2K^d-restricted antigenic peptide, the mouse $GPC3_{298-306}$ (EYILSLEEL) peptide, could be recognized by mouse CD8$^+$ CTLs. In addition, these CTLs rejected tumor expressing mouse GPC3 both *in vitro* and *in vivo* (10). Because the structural motifs of peptides bound to HLA-A24 (*A*2402*) and mouse H-2K^d are similar, we investigated whether the GPC3 peptide was also useful as a cancer immunotherapy modality for HLA-A24$^+$ HCC patients. The gene frequency of HLA-A24 (*A*2402*) is relatively high in Asian populations, especially in the Japanese, whereas it is low in Caucasians. On the other hand, The gene frequency of

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HLA-A2 (*A*0201*) is high among various ethic groups, including both Asians and Caucasians (11). Therefore, it is suggested that the HLA-A2-restricted and GPC3-derived CTL epitopes might be very useful for the immunotherapy of many patients with HCC and melanoma all over the world. In the present study, we identified human GPC3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 or HLA-A24-restricted epitope peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMC) of patients with HCC.

Materials and Methods

Mouse. HLA-A2.1 (HHD) Tgm; H-$2D^{b-/-}\beta 2m^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1 α2)-H-$2D^b$ (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France (12, 13) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan).

Patients, blood samples, and cell lines. Blood samples from patients with HCC were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients in Kumamoto University Hospital from April to September 2005. Human liver cancer cell lines, SK-Hep-1 and T2-A0201 (a TAP-deficient and HLA-A*0201-positive cell line; refs. 14, 15), were provided by Kyogo Ito of Kurume University. Human liver cancer cell lines HepG2 and HuH-7 endogenously expressing GPC3, and $GPC3^-$ colon cancer cell line SW620, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). C1R-A*2402 (an *HLA-A*2402* transfectant of C1R cells expressing a trace amount of HLA class I molecule; ref. 15) were generous gifts from Dr. Masafumi Takiguchi. The expression of HLA-A2 and HLA-A24 in these cell lines were examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 and anti-HLA-A24 mAb (One Lambda, Inc., Canoga Park, CA), respectively, in order to select target cell lines for CTL assays. The origins and HLA genotypes of these cell lines have been described elsewhere (16, 17). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS.

Induction of GPC3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human GPC3-derived peptides (purity >90%) sharing the amino acid sequences with mouse GPC3 and carrying binding motifs for *HLA-A*0201*-encoded molecules, were identified using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and we purchased a total of nine peptides carrying HLA-A2 (*A*0201*) binding motifs (Table 1) from Biologica (Tokyo, Japan). The immunizations of mice with peptides were done as previously described (7). In brief, bone marrow (BM) cells (2×10^6) from HLA-A2.1 (HHD) Tgm were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) and 2ME (0.8 ng/mL) for 7 days in 10-cm plastic dishes, and these BM-dendritic cells (DC) were pulsed with the mixture of GPC3 peptides carrying HLA-A2 binding motifs (1 μmol/L for each peptide) at 37°C for 2 hours. We primed the HLA-A2.1 (HHD) Tgm with this syngeneic BM-DC vaccine (5×10^5/mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and $CD4^-$ spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production by $CD4^+$ spleen cells cocultured with the BM-DC. The $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DC (2×10^5/well) pulsed with each peptide *in vitro*. Then, 6 days later, the frequency of cells producing IFN-γ/2×10^4 $CD4^-$ spleen cells upon stimulation with syngeneic BM-DC (1×10^4/well), pulsed with or without each peptide, was assayed in an enzyme-linked immunospot (ELISPOT) assay as previously described (18).

Induction of GPC3-reactive human CTLs. We isolated PBMCs from the heparinized blood of HLA-$A24^+$ and/or HLA-$A2^+$ Japanese patients with HCC or healthy donors by means of Ficoll-Conray density gradient centrifugation, and peripheral monocyte-derived DCs were generated as described previously (19, 20). $CD8^+$ T cells were isolated using CD8 microbeads (Miltenyi Biotec) from the PBMC of the same donors, and thereafter, peptide-reactive $CD8^+$ CTLs were generated (19, 20). Five days after the last stimulation, the cytotoxic activities of the CTLs were measured by a ^{51}Cr release assay.

CTL responses against cancer cell lines. CTLs were cocultured with each cancer cell line as a target cell (5×10^3/well) at the indicated effector/target ratio and ^{51}Cr release assay was done as described (21). The blocking of HLA-class I or HLA-DR was done as follows. Before the coculture of CTLs with a cancer cell line in a ^{51}Cr release assay or ELISPOT assay, target cancer cells were incubated for 1 hour with 10 μg/mL anti-class I mAb W6/32 or 10 μg/mL anti-HLA-DR mAb, H-DR-1, and then the effects of mAbs on either the cytotoxic activity or production of IFN-γ by CTLs were examined as reported previously (22).

Histologic and immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144-152}$ peptides and the staining of

Table 1. GPC3-derived peptides conserved between human and mouse GPC3 and predicted to be bound to HLA-A2 (*A*0201*)

A2-binding peptide	Position	Subsequence residue listing	HLA-A2 binding score*
GPC3A2-1	44-52	RLQPGLKWV	879
GPC3A2-2	102-110	FLIIQNAAV	319
GPC3A2-3	144-152	FVGEFFTDV	828
GPC3A2-4	155-163	YILGSDINV	162
GPC3A2-5	169-177	ELFDSLFPV	1055
GPC3A2-6	254-268	RMLTRMWYC	1259
GPC3A2-7	281-289	VMQGCMAGV	196
GPC3A2-8	326-334	TIHDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform).

apoptotic cells with terminal deoxynucleotidyl transferase-mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.[5]

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we s.c. inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated $CD8^+$ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were i.v. injected one more times on day 14. The $CD8^+$ T cells stimulated with HLA-A24-restricted $GPC3_{298-306}$ peptide or HIV (RYLRDQQLL) peptide and derived from two independent $HLA\text{-}A24^+$ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated $CD8^+$ T cells from two other $HLA\text{-}A24^+$ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated $CD8^+$ T cells from one $HLA\text{-}A2^+$ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated $CD8^+$ T cells derived from another $HLA\text{-}A2^+$ HCC patient.

Statistical analysis. The two-tailed Student's t test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (*A*0201*; Table 1). $CD4^-$ spleen cells from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that $CD4^-$ spleen cells stimulated *in vitro* with the $GPC3_{144-152}$ peptide produced the largest amount of IFN-γ in a peptide-specific manner in ELISPOT assays. These $CD4^-$ spleen cells (2×10^4/well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the $GPC3_{144-152}$ peptide, whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about $(36\text{-}23) / 2 \times 10^4 = 0.065\%$ of $CD4^-$ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in $CD4^-$ spleen cells stimulated with the $GPC3_{144-152}$ peptide pulsed BM-DCs was $1{,}878 \pm 131$ μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 ± 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 ± 131 μm ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the $GPC3_{144-152}$ peptide-specific response of $CD4^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the $GPC3_{144-152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this $GPC3_{144-152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, $GPC3_{144-152}$, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the $GPC3_{298-306}$ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the $GPC3_{144-152}$ peptide alone ($n = 3$; data not shown). These results indicate that the $GPC3_{144-152}$ peptide-reactive $CD8^+$ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the $GPC3_{298-306}$ or $GPC3_{144-152}$ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of $HLA\text{-}A2^+$ HCC patients stimulated with the $GPC3_{144-152}$ peptide or CTLs from PBMCs of $HLA\text{-}A24^+$ HCC patients stimulated with the $GPC3_{298-306}$ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the $GPC3_{144-152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

[5] T. Torigoe, et al. Immunohistochemical analysis of HLA class I expression in tumor tissues revealed unusually high frequency of down-regulation in breast cancer tissues submitted.

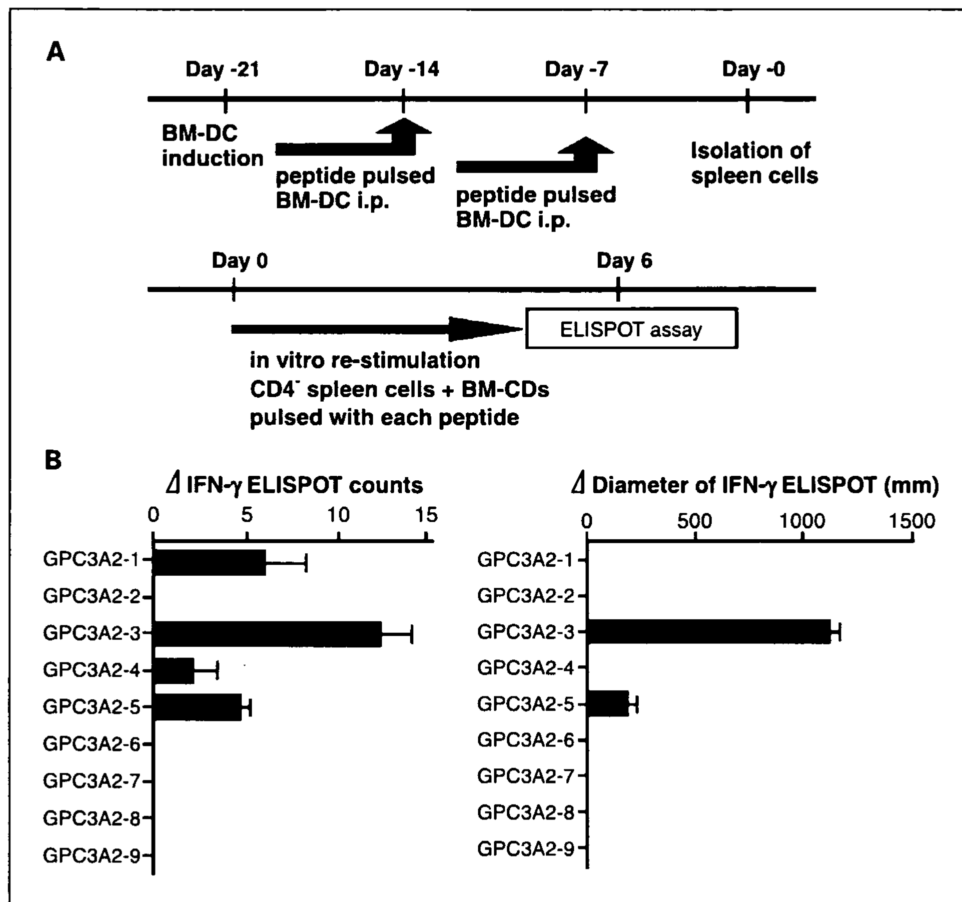

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A,* protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. *B, bar graph,* IFN-γ ELISPOT counts/2×10^4 $CD4^-$ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). *Bar graph,* summation of IFN-γ ELISPOT diameters/2×10^4 $CD4^-$ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). *Columns,* mean of triplicate assays; *bars,* SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the $GPC3_{298-306}$ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the $GPC3_{144-152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the $GPC3_{298-306}$ peptide-induced CTLs showed specific cytotoxicity against SW620/GPC3 in patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the $GPC3_{144-152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

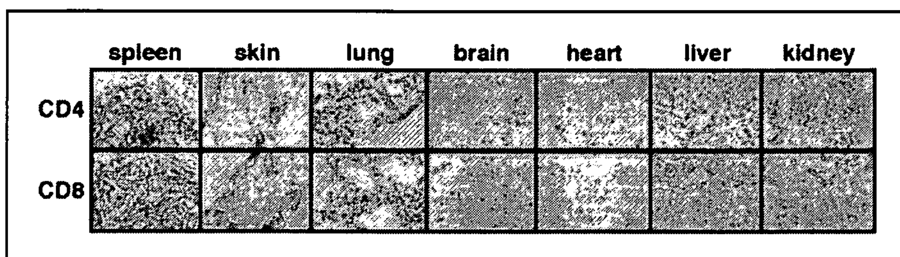

Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144-152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).

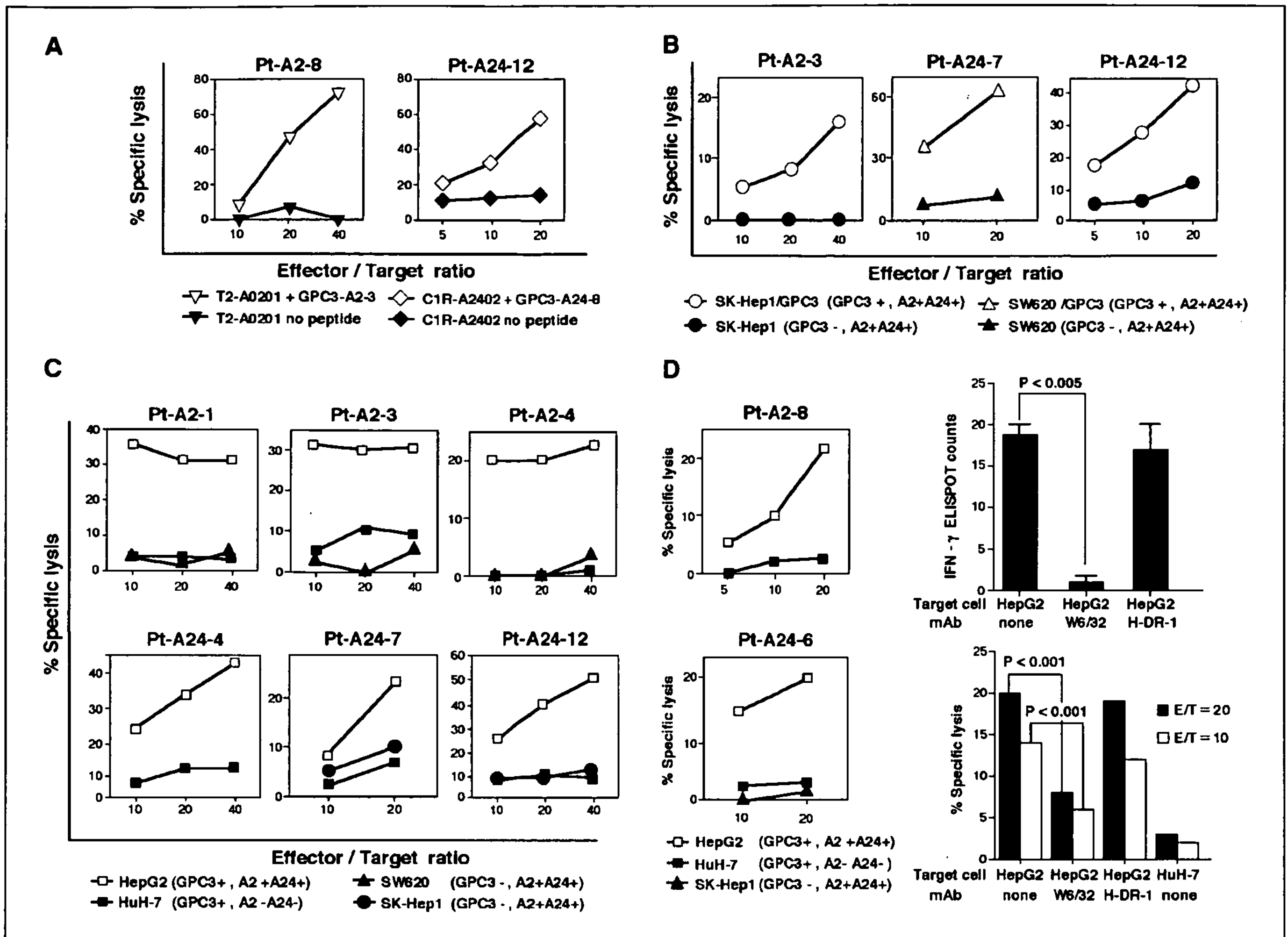

Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. *A* and *B*, GPC3 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ and/or HLA-$A24^+$ HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the $GPC3_{144-152}$ or $GPC3_{298-306}$ peptide, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the $GPC3_{298-306}$ peptide pulsed C1R-A2402 cells or T2-A0201 cells, and each unpulsed cells (*A*), or $GPC3^-$ HLA-$A2^+$, HLA-$A24^+$ HCC cell line SK-Hep-1, $GPC3^-$ HLA-$A2^+$, HLA-$A24^+$ colon cancer cell line SW620, and those cell lines transfected with the human *GPC3* gene; SK-Hep-1/GPC3 or SW620/GPC3 (*B*) were examined by a ^{51}Cr release assay. *C* and *D*, $GPC3^+$ HLA-$A2^+$, HLA-$A24^+$ HCC cell line HepG2, $GPC3^+$ HLA-$A2^-$, HLA-$A24^-$ HCC cell line HuH-7, and $GPC3^-$ tumor cell lines SW620 and SK-Hep1 were used as target cells (*left*). *Points*, percentage of specific lysis calculated based on the mean values of a triplicate assay. *D*, inhibition of cytotoxicity by anti-HLA class I mAb (*right*). After the target HepG2 cells were incubated with anti-HLA class I mAb (W6/32, IgG_{2a}) or anti-HLA DR mAb (H-DR-1, IgG_{2a}), respectively, for 1 hour, the CTLs generated from PBMCs of patient A2-8 by stimulation with $GPC3_{144-152}$ peptide (*top*) or CTLs generated from patient A24-6 using the $GPC3_{298-306}$ peptide (*bottom*) were added. IFN-γ production (*top*; IFN-γ ELISPOT assay) and cytotoxicity (*bottom*; ^{51}Cr release assay) were markedly inhibited by W6/32, but not by H-DR-1.

HLA-A24+), but not to HuH-7 (GPC3+, HLA-A2−, and HLA-A24−) or SW620 ($GPC3^-$, HLA-A2+, and HLA-A24+) in patients A2-1, A2-3, and A2-4. Similarly, we could generate GPC3-reactive CTLs by stimulation of PBMCs with the $GPC3_{298-306}$ peptide and these CTLs exhibited cytotoxic activity to HepG2, but not to HuH-7 or SK-Hep-1 ($GPC3^-$, HLA-A2+, HLA-A24+) in patients A24-4, A24-7, and A24-12.

In an HLA-class I blocking experiment, anti-HLA class I mAb W6/32 markedly inhibited the IFN-γ production stimulated with HepG2 cells in ELISPOT assay of CTLs generated from patient A2-8 by stimulation with the $GPC3_{144-152}$ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ^{51}Cr release assay of CTLs generated from patient A24-6 by stimulation with the $GPC3_{298-306}$ peptide (Fig. 3D, bottom), but anti-HLA-DR mAb, H-DR-1 did not inhibit the response of CTLs. These results clearly indicate that these CTLs recognized HepG2 in a HLA-class I–restricted manner.

As shown in Table 2, we could induce GPC3-reactive CTLs from PBMCs in ~50% of either the HLA-A2− or HLA-A24-positive HCC patients. In patients A2-6, A24-5, A24-9, and A24-11 who did not express GPC3 in tumor tissues, GPC3-reactive CTLs could not be induced from their PBMCs. Among eight HLA-A2-positive HCC patients who expressed GPC3 in HCC tissue or produced soluble GPC3 in sera, patients A2-1, A2-2, A2-3, A2-4, A2-6, A2-7, A2-9, and A2-10, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (50%). In patient A2-6, GPC3 was detected only in the serum but not in HCC tumor tissue. It was thought to be possible that the majority of GPC3 protein was secreted away in this type of HCC cell as described previously (7). Among six HLA-A24-positive patients who expressed GPC3 in tumor tissue, patients A24-1, A24-2, A24-3, A24-6, A24-10, and A24-12, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (67%). We also examined whether it was possible to

induce GPC3-specific CTLs from PBMCs isolated from healthy donors (each HLA type, $n = 3$), but we failed to generate GPC3-specific and HLA-A2- or HLA-A24-restricted CTLs even though PBMCs were stimulated with the peptides thrice *in vitro* (data not shown). These results suggest that GPC3-reactive CTLs could only be induced in patients who expressed GPC3 in tumor tissue, thus, indicating the existence of GPC3-reactive CTL precursors in patients with GPC3$^+$ HCC. We also examined whether GPC3-reactive CTLs could be generated more frequently from PBMCs isolated from HCC patients positive for serum-soluble GPC3. As shown in Table 2, the presence of serum-soluble GPC3 did not correlate statistically with the successful induction of GPC3-reactive CTLs. As a result, we could not observe the enhancement of CTL induction efficiency via possible antigen presentation of soluble serum GPC3 through HLA-class II pathways to CD4$^+$ T cells or cross-presentation through the HLA class I pathway to CD8$^+$ T cells (25, 26) in patients positive for serum GPC3.

Inoculation of the GPC3 peptide-induced CTLs reduced growth of a GPC3$^+$ human HCC tumor cell line implanted into NOD/SCID mouse. To investigate the effects of GPC3 peptide-reactive CTL inoculation into the mice implanted with the GPC3$^+$ human HCC cell line, we s.c. inoculated SK-Hep1/GPC3 cell lines positive for both HLA-A2 and HLA-A24 into NOD/SCID mice, and i.v. injected the mixture of CTLs generated from several HCC patients positive for HLA-A2 or HLA-A24 into mice implanted with SK-Hep1/GPC3 when the diameter of these tumors reached 5 × 5 mm in size as described in Materials and Methods. The CTLs injected into mice were prepared by stimulating peripheral blood CD8$^+$ T cells with HLA-A2- or HLA-A24-restricted GPC3-epitope peptides or control-irrelevant HIV peptides as described in Materials and Methods. The tumor sizes of four individual mice in each group (Fig. 4A) and mean ± SD of tumor sizes in each group (Fig. 4B) were evaluated. After 5 days from the second inoculation of GPC3 peptide-reactive CTLs, the tumor size of SK-Hep1/GPC3 was apparently reduced in comparison to the size of tumor mass implanted into NOD/SCID mice injected with control T cells or saline alone ($P < 0.01$). These results clearly indicate the efficacy of adoptive GPC3 peptide-reactive CTL transfer therapy for GPC3$^+$ tumor in mice.

Discussion

In this article, we identified HLA-A24-restricted or HLA-A2-restricted GPC3 CTL epitope peptides, and found that

Table 2. Expression of GPC3 in HCC tissue, quantification of serum-soluble GPC3, and GPC3-specific CTL induction in HCC patients

	Age	Gender	State of tumor*	GPC3 expression†	Serum GPC3‡	HLA expression§	CTL induction‖
HLA-A2 (*A*0201*) – positive patients							
Pt-A2-1	80	F	IIIa	+	+	+	+
Pt-A2-2	72	M	II	+	+	+	+
Pt-A2-3	67	F	II	ND	+	ND	+
Pt-A2-4	54	M	I	+	–	+	+
Pt-A2-5	57	M	I	ND	–	ND	–
Pt-A2-6	66	M	I	–	+	–	–
Pt-A2-7	54	M	IIIa	+	–	+	–
Pt-A2-8	73	M	II	ND	–	ND	+
Pt-A2-9	68	F	IIIa	+	–	+	–
Pt-A2-10	54	M	II	+	+	+	–
HLA-A24 (*A*2402*) -positive patients							
Pt-A24-1	60	M	IVa	+	+	+	+
Pt-A24-2	57	M	IVa	+	+	+	–
Pt-A24-3	75	F	IIIa	+	+	+	+
Pt-A24-4	59	M	IIIa	ND	–	ND	+
Pt-A24-5	52	M	IVb	–	–	+	–
Pt-A24-6	65	M	I	ND	+	ND	+
Pt-A24-7	61	M	I	ND	–	ND	+
Pt-A24-8	74	M	II	ND	–	ND	–
Pt-A24-9	59	M	IVb	–	–	–	–
Pt-A24-10	69	M	IVa	+	+	+	–
Pt-A24-11	72	M	II	–	–	+	–
Pt-A24-12	61	M	IIIa	+	+	+	+

Abbreviations: F, female; M, male; ND, not determined.
*Tumor-node-metastasis classification.
†Positive (+) or negative (–) staining of tumor cells in contrast with peritumor normal tissue as background staining.
‡Serum levels >106 ng/mL were evaluated as positive.
§Immunohistochemical staining of the membrane of tumor cells was evaluated as positive.
‖Specific lytic activity (≧20%) at E:T ratio = 20 against HepG2 target cells was evaluated as positive by ^{51}Cr release assay.

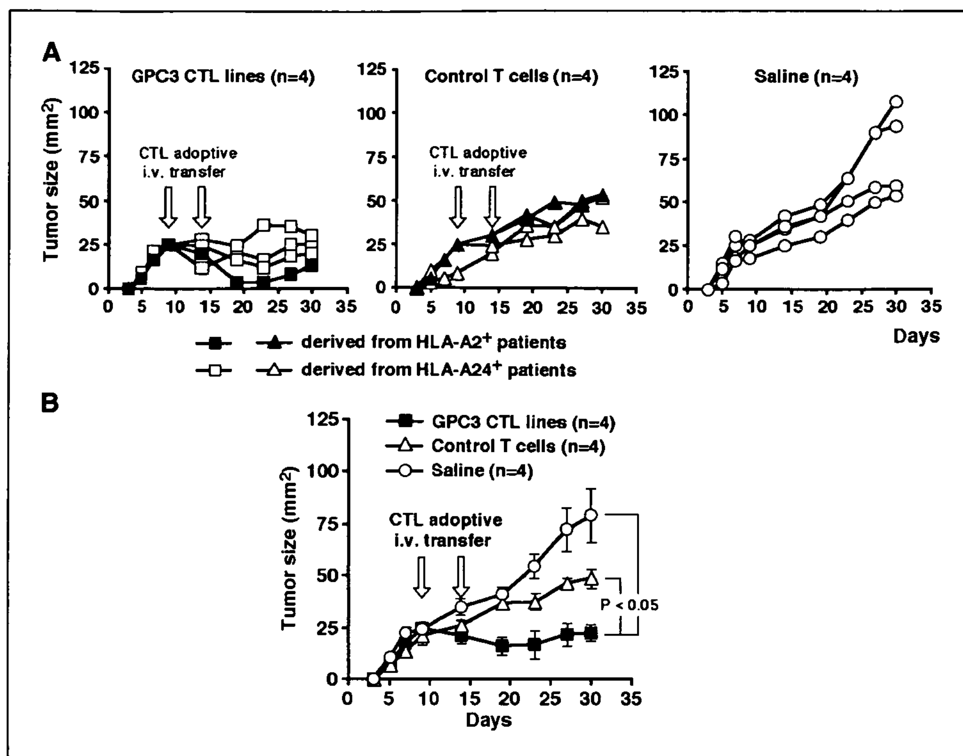

Fig. 4. Marked inhibition of growth of a GPC3-transfected human HCC cancer cell line, SK-Hep1/GPC3, engrafted into NOD/SCID mice after adoptive transfer of human CTLs induced by the GPC3 peptides. *A*, when tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs (3×10^6) reactive to HLA-A2-restricted (■) GPC3 peptide and generated from one HLA-A2$^+$ donor, or those reactive to HLA-A24-restricted (□) GPC3 peptide and pooled from two HLA-A24$^+$ donors were i.v. inoculated. On day 14, the inoculation of CTLs generated from the donors distinct from those at the first injection was repeated. The control CD8$^+$ T cells stimulated with irrelevant HLA-A2-restricted (▲) or HLA-A24-restricted (△) HIV peptides were also injected into mice as a control. Tumor volumes in NOD/SCID mice given twice on days 9 and 14 with GPC3 epitope peptide – induced CTL lines (n = 4), control CD8$^+$ T cells (n = 4), or saline alone (n = 4). Tumor size was expressed in square millimeters. *B, points,* mean tumor sizes in each group of mice; *bars,* ±SD (n = 4). Statistical significance was evaluated using t test.

GPC3-reactive CTLs could be generated from PBMCs stimulated with these peptides in ~50% of HCC patients. Vaccination based on these peptides did not induce autoimmunity in HLA-A2.1 (HHD) Tgm of a B57Bl/6 background. We previously identified the $GPC3_{298-306}$ peptide to be a CTL epitope in BALB/c mouse, and we expected that this GPC3 peptide might also be present in human CTL in a HLA-A24-restricted manner. As expected, we could generate HLA-A24-restricted and the $GPC3_{298-306}$ peptide-reactive human CTLs in this study. As a result, BALB/c mice may be useful for identifying HLA-A24-restricted CTL epitopes. HLA-A2.1 (HHD) Tgm was reported to be a versatile animal model for the preclinical evaluation of peptide-based immunotherapy (12, 13). We could also find its usefulness for the identification of HLA-A2-restricted antigenic epitope in this study.

In this study, we wanted to identify the most effective major CTL epitopes derived from GPC3. As a result, we used BM-DCs derived from HLA-A2.1 (HHD) Tgm and pulsed BM-DCs with the mixture of GPC3 peptides for the vaccination of mice. Some of the peptides tested stimulated the weak response of CTLs in an ELISPOT assay, and these peptides might also be useful for future analysis. It was recently reported that peptides having a weak affinity to MHC, which could not be predicted by a BIMAS system, could induce peptide-reactive CTLs with a cytotoxic activity (27). To search for more peptides that can be applicable for immunotherapy, it may be necessary to check these minor CTL epitopes in the future. In this study, the GPC3-derived peptides predicted to have high binding affinity to HLA-A2 molecules and having the amino acid sequences conserved between human and mouse GPCs were selected for the analysis. When we analyzed the amino acid sequence of human GPC3 protein, all of the top 28 human GPC3 peptides having high binding scores (>100) to HLA-A2 molecules shared the same amino acid sequences with mouse GPC3. Therefore, it is unlikely that we excluded many candidates of human GPC3-derived and HLA-A2-restricted CTL epitopes from the analysis by selecting the peptides having amino acid sequences shared between human and mouse GPC3. Furthermore, we have to consider the differences in the T cell repertoire in mice and humans. Thereby, we may miss GPC3 peptides recognized by human CTLs but not by mouse CTLs.

Considering ideal immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that could not be lost by tumor cells through immunoediting (28, 29). Recently, Capurro et al. reported that GPC3 is involved in the carcinogenesis and proliferation of HCC via regulation of noncanonical Wnt signals (30). Therefore, it may be possible that tumor cells cannot lose the GPC3 expression in order to continue to grow. Furthermore, according to an immunohistochemical analysis of the expression of HLA-class I molecules using newly developed specific mAb, EMR 8-5,[5] we found that almost all HCC cells expressed HLA-class I as far as we could examine (Table 2). For these reasons, we think that GPC3 is a very useful candidate as a target tumor antigen for the immunotherapy of HCC. We and others previously reported that the expression of GPC3 in HCC was detected from an early stage and the quantification of the soluble GPC3 protein in sera was useful for a diagnosis of HCC at an early stage (5, 7). As a result, GPC3-based immunotherapy might be able to prevent the appearance of HCC in patients with hepatitis B or C-based liver cirrhosis.

In this study, we found that it is possible to induce GPC3-reactive CTLs by the stimulation of PBMCs with the two major GPC3 epitopes *in vitro* in 50% of the HCC patients having an appropriate *HLA-class I* allele. However, it is necessary to investigate more patients to estimate the probability of a

successful induction of GPC3-reactive CTLs in HCC patients. We intended to know whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into tumor tissue of these patients, therefore, we investigated the seven index cases; patients A2-10, A24-1, A24-2, A24-4, A24-9, A24-11, and A24-12, to see whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into the tumor tissue of these patients. In three patients, A24-1, A24-4, and A24-12, who could generate GPC3 peptide-reactive CTLs, patient A24-12 recurred at 6 months after operation. In four patients, A2-10, A24-2, A24-9, and A24-11, who failed to induce GPC3-peptide-reactive CTLs, patient A24-9, whose HCC did not express GPC3, recurred at 6 months after operation, and patient A24-2 recurred at 3 months after operation and died 3 months after recurrence. These three recurred patients had extremely strong tumor invasion to the vasculature. Therefore, it was difficult to evaluate the correlation between the positive CTL response and clinical improvement at the present stage, and we have to increase the number of patients investigated and to do further statistical analyses on these relationships. In patients who could be examined for the infiltration of CD8-positive cells into their tumor specimens and for the existence of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in tumor tissue, patients A2-10, A24-1, A24-2, and A24-9, there was no strong correlation between the positive GPC3 peptide-reactive CTL response and for the existence of CD8-positive or terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in the tumor tissues (data not shown). As shown in Fig. 4, we observed a regression of the tumor masses in NOD/SCID mice implanted with SK-Hep1/GPC3 and transferred i.v. with the GPC3 peptide-reactive CTLs in comparison to the mice injected with control $CD8^+$ T cells or saline alone. Although the regression of tumor growth was observed for 2 weeks after the second transfer of CTLs, the tumors began to enlarge again after that period. We thought it was important to continue the transfer of CTLs again and again to obtain continuous regression of the GPC3-expressing tumor. These data suggest that the adoptive i.v. transfer of GPC3-reactive CTLs into mice bearing $GPC3^+$ tumors was useful to inhibit tumor growth in the mouse tumor model.

In addition, it is most important to confirm the usefulness of GPC3-specific *in vivo* cancer immunotherapy in patients with HCC. Investigation of the presence of GPC3-specific CTLs in patients with melanoma are also eagerly awaited. We previously reported that DC differentiated *in vitro* from mouse embryonic stem cells transfected with the mouse *GPC3* gene (24, 31) induced protective immunity against mouse melanoma cell line B16 F10 (32). We are now preparing a translational study of GPC3-based immunotherapy to reduce the risk of recurrence in HCC patients treated surgically. We will try to use the GPC3 epitope peptides identified in this study first, whereas in the second phase, we will make a trial of the peptide-pulsed DC vaccine. We expect that GPC3-based immunotherapy may be a novel treatment strategy that could potentially help to prevent the appearance, advance, and/or recurrence of HCC and melanoma.

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