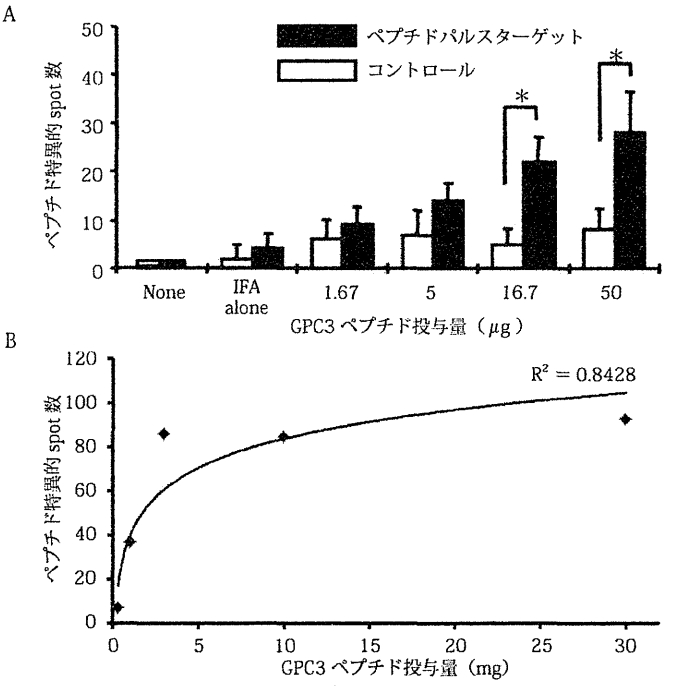
るかといった検討や臨床試験で用いる2種類のGPC3ペプチドと共に投与する至適アジュバントについてマウスを用いた検討を行った[19]。BALB/cマウスを用いて，ペプチド単独群，不完全フロイントアジュバント（incomplete fluid adjuvant：IFA）との併用群，CpG併用群，α-GalCel併用群，アルミニュウム併用群の5群で比較したところ，IFAとの併用投与群においてのみ，GPC3特異的なキラーT細胞（CTL）が誘導された。ペプチド単独では無効で，IFAと混合すると有効になることを証明し，臨床試験ではペプチドとIFAの混合物を投与することとした。我々が行ったマウスの実験では，ペプチド投与量に依存して強い免疫を誘導できるとの結果に至った（図3A)。マウスで今回投与した1回あたりの最大量は50 μgであったが，単純に体重換算すると，マウスでの50 μgはヒトでの100mgに相当し，コストも膨大となるばかりか，その溶液を皮内注射するとなれば1回に数十ヶ所も注射しなければならない量であり，現実的には不可能な量であった。そこで臨床第I相試験では，1回投与量を0.3mgから段階的に増量とし，安全性を確認しながら容量を増やしていく設定にし，免疫学的モニタリングにより次相の至適投与量を決める方針とした。また医師法のもとで行われる自主研究ではあるが，臨床試験で投与するペプチドは，ヒトの体内に投与されても副作用が出ないように，製造過程が全て記録され，可能な限り不純物が含まれていない事が詳細に検査された高純度のものを入手する必要があった。購入したペプチドは，このような規格を満たすものであり，米国の医薬品製造ライセンスを有し，cGMP施設として承認された工場で臨床研究用原薬としてのペプチドを大量生産できる米国会社にて依頼生産され，かつ厳重な管理下での製造および品質管理の過程を経ている。ペプチドを合成し提供する会社が保証するGMPはあくまで品質の保証であり，安全性は担保されない。そのため，我々はGPC3ペプチドのマウスを用いた単回皮下投与毒性試験を国内民間企業に依託し，報告書を作成した。GPC3ペプチド（GPC3ペプチドA24，GPC3ペプチドA2）を6000 μg/kg及び60000 μg/kgの用量でマウスに単回皮下投与した時，媒体に起因すると考えられる投与部位皮膚の痂皮形成が全群に認められたが，GPC3ペプチドに起因すると考えられる変化は認められないことから，GPC3ペプチドの毒性量は60000 μg/kgを上回るものと考えられた。即ち，ヒトの体重を50kgとすると本臨床試験で推奨投与量と判断された3mgは60 μg/kgの用量であるが，その100倍量，1000倍量に相当する6000 μg/kg及び60000 μg/kgの用量でマウスに投与しても安全である事がいえる根拠となった。



A. BALB/C マウスを用いた検討では，投与量依存性の免疫反応を認めた。Motomura Y et al, 2008 より改変引用[19]。
B. GPC3 ペプチドワクチン臨床第 I 相試験でも投与量依存性を認める。Sawada Y et al, 2012 より改変引用[9]。

図3　ペプチド投与量と IFN-γ ELISPOT assay におけるスポット数の関係

3.2 GPC3 ペプチドワクチン臨床第 I 相試験[8,9]

国立がん研究センター東病院において，進行肝細胞がん 33 例を対象に GPC3 ペプチドワクチン第 I 相臨床試験を 2007 年 2 月に開始し，2009 年 11 月に完了した。0.3mg, 1mg, 3mg, 10mg, 30mg と投与量を増量して投与した結果，容量制限毒性（dose limiting toxity：DLT）は，1 例も認めず，最大耐用量（maximum tolerance dose：MTD）の決定は困難であった。30mg 投与の 1 例に PR の臨床効果が認められたことや，免疫学的モニタリングの結果において用量依存性が認められた（図 3B）ことからは高用量投与の優位性が示唆された。しかし 30mg は，3mg 投与の 10 倍量の 6ml もの量を皮内に投与するため投与手技が煩雑な上，患者の苦痛も大きく，投与部位の発赤・硬結が同じ grade 1 でも明らかに大きかったことから，その臨床効果と合わせて考えると次相の GPC3 ペプチドワクチンの推奨投与量は 3mg が妥当であると判断した。当時は手探りの感があったが，FDA ガイダンスの提言どおり MTD は見られず，至適投与量は投与部位の解剖学的な問題に起因するといった結果であった。

この臨床第 I 相試験では，安全性の確認，腫瘍マーカー低下などの臨床効果のほか，IFN - γ ELISPOT 法による末梢血中ペプチド特異的 CTL の頻度の増加の検出，ワクチン後の腫瘍の生検を行い，ワクチン前の腫瘍内には浸潤していなかった CD8 陽性の CTL がワクチン後の腫瘍内に多数浸潤している像も観察できたなどの免疫学的有効性も確認できた。臨床試験で抗腫瘍免疫応答のモニタリングが可能となっていることは，間違いがないと考えている。

3.3 国立がん研究センター東病院における GPC3 ペプチドワクチン臨床試験の計画

がんワクチン療法標準化へ向けては，第 II 相臨床試験のデザインこそが大事だと考えており，誰もが納得するような有効性の証明が重要と考えている。現在，GPC3 ペプチドワクチンに関して手術やラジオ波焼灼療法（RFA）などの肝細胞がん根治的治療後の再発予防効果を検証する第 II 相臨床試験を実施中である。根治治療後 1 年間にワクチンを計 10 回投与する計画としたが，これは臨床第 I 相試験の結果から，ワクチンを継続投与した症例で末梢血中の CTL が持続的に観察される傾向が強かったことより 10 回という回数を設定した。また早期に次相のランダム化試験につなげることを前提とし，1 年，2 年再発率をエンドポイントとした単群早期第 II 相試験であり，解析予定症例数は 40 例としている。この 40 症例の算出は，次相の臨床試験を行う根拠を得ることを目的に，ヒストリカルコントロールを対照群と設定して行った。初回病変を切除手術もしくはラジオ波焼灼療法で根治的に治療できて当院で経過観察できた症例群を調査すると 1 年，2 年再発率は，切除手術とラジオ波焼灼療法はほぼ同等で，各 35-45%，60-70%であったが，以下の計算では，1 年再発率 40%，2 年再発率 60%と仮定する。

得られた結果が現状の 1 年再発率 40%よりは統計学的に有意（危険率 5%，信頼度 95%）なるための必要最小症例数を以下の式で算出することで有効症例数を検討した。

Entry N 人，一年後 n 人無再発，m 人：再発 $n + m = N$

無再発率とその 95% 信頼区間

無再発率 $p\ (= n/N)$, $q = 1 - p$

無再発率 p の 95% 信頼区間 $= (p - 1.96 * \mathrm{sqrt}\ (pq/N)$, $p + 1.96 * \mathrm{sqrt}\ (pq/N)$

1 年再発率 40%を半分の 20%に落とす効果を持っていると仮定した場合，必要最小症例数は $N = 16$ となるが，さらに検出力を 80%以上にあげるために $N = 40$ とした。

40 例の場合，40%の 16 例が再発するところを，20%以下の 8 例以下に抑えることができれば，再発抑制効果として有意な差となる（$P < 0.01$）。この場合，40 人中の 8 人（20%）にメリットのある治療であることが主張でき，それは十分意味があると考えられる。仮に再発が 10 人（25%）に認められた場合も，再発抑制効果として有意な差となり（$P < 0.05$），この場合も 40 人中の 6 人（15%）にメリットのある治療であることが主張でき，それも意味があると考えられる。以上より，40 人で 1 年再発率を用いた中間解析を行い，再発が 8 人以下にしか認められなかった場合，明らかに有効と判断して次相ランダム化の臨床試験に進む根拠とする設定とした。

またペプチドワクチン療法の POC は，投与後に血液中にペプチド特異的 CTL が増えるかどうか，さらにその CTL が実際がんの組織の中に浸潤するかどうかを証明することである。我々は，GPC3 ペプチドワクチン投与により末梢血中に GPC3 特異的 CTL が誘導できるという十分な証拠を，前述の第 I 相臨床試験から得ることができたが，ワクチン投

与後の腫瘍浸潤 CD8 陽性 T リンパ球に関して，十分な解析はできていない。現在，ワクチン投与後の腫瘍浸潤リンパ球の解析のため，進行肝細胞がん患者を対象とし GPC3 ペプチドワクチン療法前後で全例に肝生検を行う臨床試験を行っている。この試験のプライマリーエンドポイントは，ペプチドワクチン投与前後の生検組織検体における CD8 陽性 T 細胞の腫瘍内浸潤の増加の有無としている。ペプチドワクチンは，今後は国内でも製薬企業での治験での実施が見込まれるが，このようながん免疫療法の概念実証となる可能性を持った探索的臨床研究は，アカデミアで行う価値があると考えている。

進行肝細胞がん患者を対象とした分子標的薬ソラフェニブ（ネクサバール®）と GPC3 ペプチドワクチン療法併用の有効性を評価するランダム化臨床第 II 相試験（医師主導臨床試験）の計画は，種々の事情によりとん挫した。計画では臨床第 II 相試験での予定症例数は，ヒストリカルコントロールに対して期待される生存期間の延長を有意な差として検出するための必要最小症例数としてワクチン併用群で 40 例と設定した。OS をプライマリーエンドポイントとしているため，選択バイアスの問題も考慮し，ソラフェニブ単独群の生存期間中央値，ワクチン併用群の 95%信頼区間を確認するために，ワクチン併用群の予定症例数に合わせて，ソラフェニブ単独群の予定症例数も 40 例に設定した。この症例数はあくまでも GPC3 ペプチドワクチン療法の有効性を検証する第 II 相試験として，次の第 III 相試験を計画する意義があるか検証するためのものであり，無作為比較試験としてソラフェニブ単独群との有意差を検証するためのものではなかった。参考として，第 III 相試験として無作為比較試験を行う場合の理想的な必要症例数を算定すると，α エラーを 0.05，β エラーを 0.2 と仮定すれば，両群あわせて約 320 例となる。

アカデミアで基礎研究，臨床第 I 相試験まで完了し，以降の医師主導臨床試験も継続しているが，創薬に向けては製薬企業の治験が進んでいくことに今後シフトする予定である。

おわりに

通常，抗がん剤や分子標的薬などの開発において，大規模な治験に行くためには，大金をつぎ込んだ Phase 0 の毒性試験などの実施が必要であるが，ペプチドワクチンのようなものは従来の抗がん剤の Phase 0 より省略できるところも多いと考えられる。多くの臨床試験が安全性を証明している。PMDA がどれだけ規制緩和してくれるかもポイントである。

日本でのがんワクチンの医師主導の臨床試験は，資金面，体制面とも不足しており，そこで有望そうな結果が出たとしても，しっかりとしたエビデンスを証明できるにいたっていなかったため，製薬企業が開発に乗り出さず，大規模にスピード感を持って進まなかった。国家プロジェクトでできるだけ可能性のある多くの臨床試験に対してしっかりとエビデンスを構築できるような惜しみない支援をし，製薬企業が開発意欲をそそる臨床試験を拾い上げられるような仕組みをつくる必要がある。また患者や紹介する医師側に，現在登録できる臨床試験のリストがいつでも閲覧できるような仕組みも必要である。

今後アカデミアが開発したものを医師主導試験で安全性と有効性をある程度示せないと製薬企業が開発に乗り出さないという現状を，日本でも最初から企業が参画して治験でやる時代に変えられるのかどうか，我々の施設もまずはそのモデルケースづくりにも挑戦していきたい。

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HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells

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Glypican-3 (GPC3) is an onco-fetal antigen that is overexpressed in human hepatocellular carcinoma (HCC), and is only expressed in the placenta and embryonic liver among normal tissues. Previously, we identified an HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide that can induce GPC3-reactive CTLs without inducing autoimmunity in HLA-A2 transgenic mice. In this study, we carried out a phase I clinical trial of HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in 14 patients with advanced HCC. Immunological responses were analyzed by *ex vivo* γ-interferon enzyme-linked immunospot assay. The frequency of $GPC3_{144-152}$ peptide-specific CTLs after vaccination (mean, 96; range, 5–441) was significantly larger than that before vaccination (mean, 6.5; range, 0–43) ($P < 0.01$). An increase in the $GPC3_{144-152}$ peptide-specific CTL frequency was observed in 12 (86%) of 14 patients after vaccination. Additionally, there was a significant correlation between the maximum value of $GPC3_{144-152}$ peptide-specific CTLs after vaccination and the dose of the peptide injected ($P = 0.0166$, $r = 0.665$). Moreover, we established several $GPC3_{144-152}$ peptide-specific CTL clones from PBMCs of patients vaccinated with $GPC3_{144-152}$ peptide by single cell sorting using Dextramer and CD107a antibody. These CTL clones had high avidity (the recognition efficiency showing 50% cytotoxicity was 10^{-10} or 10^{-11} M) and could recognize HCC cell lines expressing GPC3 in an HLA-class I-restricted manner. These results suggest that $GPC3_{144-152}$ peptide vaccine can induce high avidity CTLs capable of killing HCC cells expressing GPC3. This trial was registered with University Hospital Medical Information Network number 000001395. (*Cancer Sci* 2011; 102: 918–925)

In peptide-based vaccine trials, occasional marked clinical regressions of melanoma have been observed after peptide vaccination; however, tumor regressions have not correlated well with T cell responses measured in peripheral blood lymphocytes.[1–3] This may be because the clinical response to a vaccine was unrelated to the immune response to that vaccine or due to inadequate immune response monitoring. Moreover, vaccination with synthetic peptides has occasionally induced ineffective CTL responses due to various mechanisms.[4–9] When evaluating T cell response to peptide vaccines, it is important to confirm that the peptide is presented naturally on cancer cells and that responding CTLs lyse human cancer cells.

Glypican-3 (GPC3) is specifically overexpressed in human hepatocellular carcinoma (HCC).[10] The expression of GPC3 was correlated with a poor prognosis in HCC patients.[11] Moreover, GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several studies with mice.[12–14] We identified HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive CTLs without inducing autoimmunity,[15] and reported a preclinical study using a mouse model with a view to designing an optimal schedule for the clinical trials of a GPC3-derived peptide vaccine and showed dose-dependency in the immunizing effect of the peptide vaccine.[16]

In this study, we completed the phase I clinical trial of a GPC3-derived peptide vaccine for 30 patients with advanced HCC (manuscript in preparation). Among them, 16 patients had the *HLA-A24* gene and 14 had the *HLA-A2* gene. Here, we describe the immunological evaluation of HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in a phase I trial involving 14 patients. We highlight three important points: (i) HLA-A2-restricted $GPC3_{144-152}$ peptide is immunogenic in advanced HCC patients; (ii) dose-dependent effects of $GPC3_{144-152}$ peptide vaccine; and (iii) establishment of CTL clones showing not only high avidity but also natural antigen-specific killing activity against HCC cells.

Materials and Methods

Patients. Fourteen patients with advanced HCC were injected with HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide vaccine at the National Cancer Center Hospital East (Kashiwa, Japan). *HLA-A2* gene-positive status was determined by genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan). All patients gave written informed consent before entering the study. The profiles of the 14 patients are summarized in Table 1. This study was approved by the Ethics Committee of the National Cancer Center, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment protocol. Vaccinations with GMP grade peptide, $GPC3_{144-152}$ (FVGEFFTDV) (American Peptide Co., Sunnyvale, CA, USA) emulsified with incomplete Freund's adjuvant (Montanide ISA-51 VG; Seppic, Paris, France) were carried out intradermally three times at 14-day intervals. Five incremental dose levels at 0.3, 1, 3, 10, and 30 mg/body were planned for the peptide administration.

Preparation of PBMCs. Peripheral blood (30 mL) was obtained from each patient at times designated in the protocol (before the first vaccination and 2 weeks after each vaccination) and centrifuged using a Ficoll–Paque gradient.

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Table 1. Summary of profiles of 14 patients with advanced human hepatocellular carcinoma who participated in this study, with their clinical and immunological responses before and after vaccination with HLA-A2-restricted $GPC3_{144-152}$ peptide

Pt.	HLA	Age (years)	Sex	Stage	Dose of peptide (mg)	Clinical response†	GPC3-specific CTLs‡		
							Pre	Post	Change
A2-1	A*02:06/A*02:07	67	M	IV	0.3	SD	43	40	–
A2-2	A*02:01	62	M	IIIA	0.3	PD	0	18	+
A2-3	A*02:01	55	M	IIIA	0.3	SD	1	10	+
A2-4	A*02:01	68	F	IIIC	1.0	SD	16	15	–
A2-5	A*02:01	72	M	IIIA	1.0	SD	16	101	+
A2-6	A*02:01/A*02:06	62	M	II	1.0	PD	0	23	+
A2-7	A*02:01	67	F	IV	3.0	SD	0	23	+
A2-8	A*02:01	58	M	IIIA	3.0	SD	0	101	+
A2-9	A*02:01	52	M	IV	10.0	SD	1	100	+
A2-10	A*02:01	70	M	IV	10.0	PD	0	5	+
A2-11	A*02:01	68	M	II	10.0	PD	1	125	+
A2-12	A*02:07	75	F	IV	30.0	PR	11	196	+
A2-13	A*02:06	52	M	IV	30.0	PD	2	151	+
A2-14	A*02:01	67	M	IV	30.0	PD	0	441	+

†The clinical response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines. ‡Peripheral blood was taken from each patient before and after vaccination, and glypican-3 (GPC3)-specific CTLs were measured by *ex vivo* γ-interferon enzyme-linked immunospot assay. F, female; M, male; PD, progressive disease; PR, partial response; Pt., patient; SD, stable disease; +, increase; –, decrease.

***Ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis.** ELISPOT assay for the detection of antigen-specific IFN-γ producing T cells was carried out using the BD ELISPOT kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocols. In brief, non-cultured PBMCs (5×10^5 cells/well) were added to plates in the presence of 10 μg/mL peptide antigens and incubated for 20 h at 37°C, 5% CO_2. The GPC3 antigen was HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide. The PBMCs with HLA-A2-restricted HIV_{19-27} (TLNAWVKVV) peptide were used as a negative control. The spots were automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cell lines. The human liver cancer cell line HepG2 ($GPC3^+$, HLA-A*02:01/A*24:02), SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02), the human melanoma cell line 526mel ($GPC3^+$, HLA-A*02:01), and the human colon cancer cell line SW620 ($GPC3^-$, HLA-A*02:01/A*24:02) were used as target cells. T2 (HLA-A*02:01, TAP^-) was pulsed with $GPC3_{144-152}$ peptide or HIV_{19-27} peptide at room temperature for 1 h. They were conserved in our laboratory.

Induction of $GPC3_{144-152}$ peptide-specific CTLs from PBMCs. The PBMCs were cultured (2×10^6 cells/well) with 10 μg/mL $GPC3_{144-152}$ peptide in AIM-V medium supplemented with 10% human AB serum, recombinant human interleukin (IL)-2 for 14 days.

Dextramer staining and flow cytometry analysis. The PBMCs were stained with HLA-A*02:01 Dextramer-RPE ($GPC3_{144-152}$ [FVGEFFTDV], HIV_{19-27} [TLNAWVKVV]; Immudex, Copenhagen, Denmark) for 10 min at room temperature and anti-CD8-FITC (ProImmune, Oxford, UK) for 20 min at 4°C. Flow cytometry analysis was carried out using FACSAria cell sorter (BD Bioscience).

CD107a staining and flow cytometry analysis. $CD8^+$ T cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs stimulated with $GPC3_{144-152}$ peptide for 14 days. $CD8^+$ T cells were incubated with T2 pulsed with $GPC3_{144-152}$ or HIV_{19-27} peptide and HepG2 at a 2:1 ratio for 3.5 h at 37°C. CD107a-specific antibodies (BD Bioscience) were included during the incubation period.

Generation of CTL clones. $CD8^+$ GPC3 $Dextramer^+$ or $CD107a^+$ cells were sorted using a FACSAria cell sorter and seeded in a 96-well plate (1 cell/well) and stimulated by the addition of irradiated (100 Gy) allogeneic PBMCs (8×10^4 cells/well) as feeder cells, in AIM-V medium supplemented with 10% human AB serum, IL-2 (200 U/mL), and phytohemagglutinin-P (PHA) (5 μg/mL) for 14–21 days.

Response of CTL clones against cancer cell lines. The CTL clones were cocultured with each cancer cell line as a target cell at the indicated effector/target (E/T) ratio, and cytotoxicity assay or IFN-γ ELISPOT assay was carried out. Blocking of HLA-class I or HLA-A2 was carried out as previously described.[15]

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech). Target cells were labeled with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37°C. The labeled cells were then incubated with effector cells for 4–6 h. Fluorescence intensity was measured before and after the 4–6 h culture, and specific cytotoxic activity was calculated using the following formula: % cytotoxicity = {1– [(average fluorescence of the sample wells – average fluorescence of the maximal release control wells)/(average fluorescence of the minimal release control wells – average fluorescence of the maximal release control wells)]} × 100%.

Determination of recognition efficiency. Calcein AM-labeled T2 target cells were pulsed with a range of peptide concentrations, starting at 10^{-6} M and decreasing by log steps to 10^{-14} M. The CTL clones were incubated with T2 target cells at a 10:1 E/T ratio for 4 h. For each CTL clone, % cytotoxicity was plotted against each peptide concentration. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone.

Cold inhibition assay. Calcein AM-labeled target cells were cultured with effector cells in a 96-well plate with cold target cells. T2 target cells, which were prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide, were used as cold target cells.

RNA interference. Small interfering RNAs specific for human GPC3 were chemically synthesized double-strand RNAs (Invitrogen, Carlsbad, CA, USA). A non-silencing siRNA, AllStras Neg. Control siRNA, was obtained from Qiagen (Valencia, CA, USA). The GPC3-specific siRNA sequence used in this study was: 5′-GGAGGCUCUGGUGAUGGAAUGAUAA-3′. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols.

Statistical analysis. Student's *t*-test was used to determine statistically significant differences between the two groups.

Correlation between the frequency of GPC3-specific CTLs and the dose of the peptide injected was analyzed using Spearman's rank correlation coefficient. Data from the ELISPOT assay using siRNA were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set as $P < 0.05$.

Results

Analysis of $GPC3_{144-152}$ peptide-specific CTLs in PBMCs of vaccinated patients. To analyze immune responses in the 14 patients vaccinated with $GPC3_{144-152}$ peptide, we evaluated the $GPC3_{144-152}$ peptide-specific immune responses by *ex vivo* IFN-γ ELISPOT assay. The representative data of patient A2-12 on changes in the frequency of $GPC3_{144-152}$ peptide-specific CTLs before and after vaccination are shown in Figure 1(a). The frequencies of $GPC3_{144-152}$ peptide-specific CTLs were 11 and 196 of 5×10^5 PBMCs at pre- and post-vaccination, respectively. The results of the comparison of the frequency of $GPC3_{144-152}$ peptide-specific CTLs before vaccination and after vaccination in all patients are shown in Table 1 and Figure 1(b). $GPC3_{144-152}$ peptide-specific CTLs were clearly detected in four and 14 of the 14 patients at pre- and post-vaccination, respectively. The frequency of $GPC3_{144-152}$ peptide-specific CTLs after vaccination (mean, 96; range, 5–441) was significantly larger than that before vaccination (mean, 6.5; range, 0–43) ($P < 0.01$). An increase in $GPC3_{144-152}$ peptide-specific CTLs was found in 12 (86%) of the 14 patients, except in two cases (patients A2-1 and A2-4). These results suggest that $GPC3_{144-152}$ peptide vaccination can induce an increase in $GPC3_{144-152}$ peptide-specific CTLs in HCC patients. Moreover, we compared the frequency of $GPC3_{144-152}$ peptide-specific CTLs after vaccination for each dose of peptide injected. We found that the maximum value of $GPC3_{144-152}$ peptide-specific CTLs after vaccination was significantly correlated with the dose of the peptide injected ($P = 0.0166$, $r = 0.665$) (Fig. 1c).

Establishment of $GPC3_{144-152}$ peptide-specific CTL clones by three different methods. To further investigate the ability of $GPC3_{144-152}$ peptide-specific CTLs induced by peptide vaccination to recognize an antigen, we established CTL clones from PBMCs of three vaccinated patients (patients A2-8, A2-9, and A2-14) by three different methods (Fig. 2). A representative clone from each patient is shown. In patient A2-9 (Fig. 2a), the frequency of $GPC3_{144-152}$ peptide-specific CTLs was 50 of 5×10^5 PBMCs 1 month after the third vaccination, as determined by *ex vivo* ELISPOT assay, and 14 days after the *in vitro* stimulation with $GPC3_{144-152}$ peptide, Dextramer assay was carried out. The population of $CD8^+$ GPC3 $Dextramer^+$ cells was 2.6% of all stimulated cells. These cells were sorted to a single cell in each well of a 96-well plate. Twenty-one days after cell sorting, peptide specificity was examined by Dextramer assay. The established CTL clone was $CD8^+$ GPC3 $Dextramer^+$ cells (99.7%) which did not react with HIV Dextramer as a negative control (Fig. 2a).

We next attempted to sort from small populations of $GPC3_{144-152}$ peptide-specific CTLs without *in vitro* culture. In patient A2-14 (Fig. 2b), the frequency of $GPC3_{144-152}$ peptide-specific CTLs was 329 of 5×10^5 PBMCs 2 weeks after the third vaccination, as determined by *ex vivo* ELISPOT assay; $CD8^+$ GPC3 $Dextramer^+$ cells could be clearly detected in 0.1% of PBMCs. $CD8^+$ GPC3 $Dextramer^+$ cells were directly sorted to a single cell from PBMCs without *in vitro* stimulation. The established CTL clone was $CD8^+$ GPC3 $Dextramer^+$ cells (99.9%) which did not react with HIV-Dextramer (Fig. 2b).

Finally, to establish high avidity and tumor-reactive CTLs from a heterogeneous population, we attempted to sort the population of $CD8^+$ T cells which mobilized CD107a in response to naturally GPC3-expressing HepG2 cells. In the PBMCs from

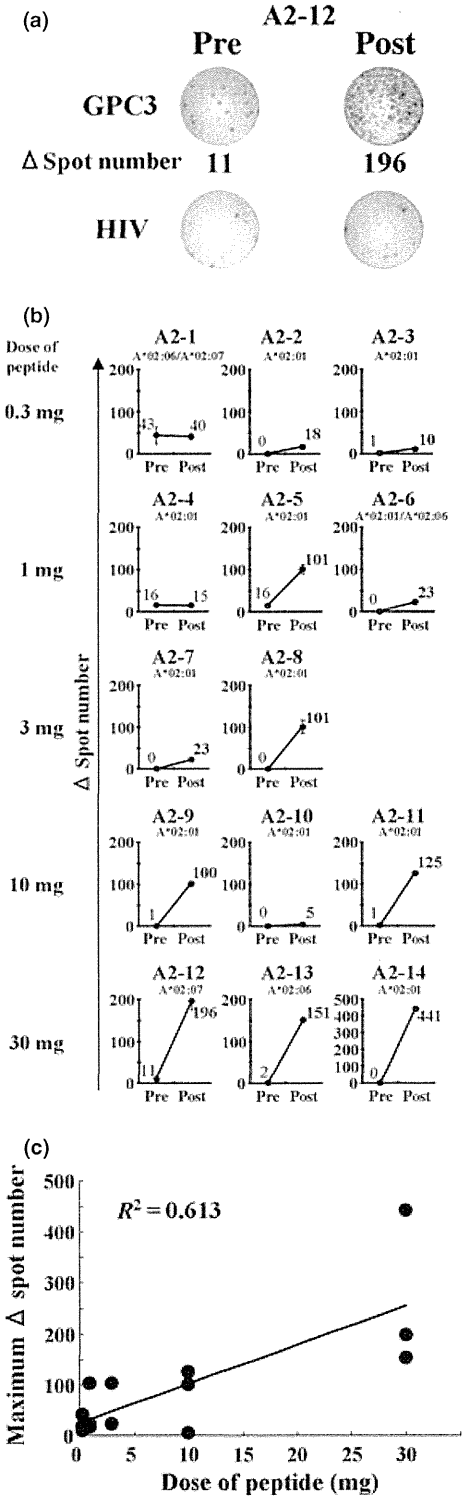

Fig. 1. Changes in the frequency of $GPC3_{144-152}$ peptide-specific CTLs before and after vaccination. Direct *ex vivo* γ-interferon enzyme-linked immunospot assay of PBMCs (5×10^5) was carried out. The Δ spot number indicates the number of $GPC3_{144-152}$ peptide-specific CTLs calculated by subtracting the spot number in a well of HIV_{19-27} peptide. (a) Representative result showing the frequency of $GPC3_{144-152}$ peptide-specific CTLs pre- and post-vaccination. (b) Changes in the frequency of $GPC3_{144-152}$ peptide-specific CTLs before and after vaccination in all patients (A2-1–14). An increase in $GPC3_{144-152}$ peptide-specific CTLs was observed in 12 (86%) of 14 patients. (c) The maximum number of $GPC3_{144-152}$ peptide-specific CTLs after vaccination was significantly correlated with the dose of the peptide injected ($P = 0.0166$, $r = 0.665$).

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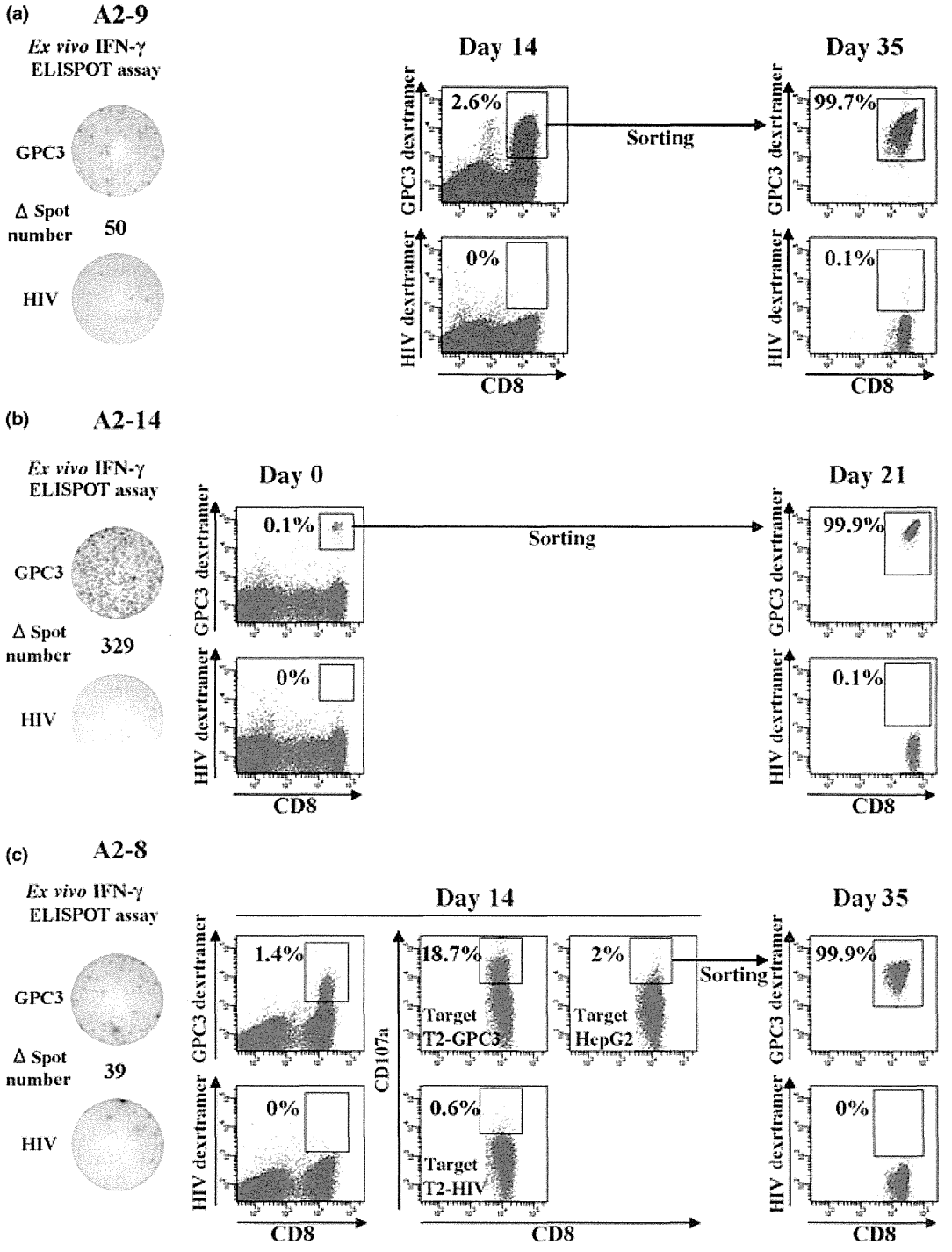

Fig. 2. Establishment of $GPC3_{144–152}$ peptide-specific CTL clones by three different methods. Left panels show the frequency of $GPC3_{144–152}$ peptide-specific CTLs in the PBMCs used, as established by *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. (a) The PBMCs of patient A2-9 were stimulated with $GPC3_{144–152}$ peptide *in vitro* for 14 days. The population of $CD8^+$ GPC3 $Dextramer^+$ cells was sorted to a single cell. (b) $CD8^+$ GPC3 $Dextramer^+$ cells were directly sorted to a single cell from PBMCs of patient A2-14 without *in vitro* stimulation. (c) The PBMCs of patient A2-8 were stimulated with $GPC3_{144–152}$ peptide *in vitro* for 14 days. $CD8^+$ $CD107a^+$ cells that reacted against HepG2 were sorted to a single cell. Right panels show Dextramer analysis of the established clones 21 days after cell sorting.

patient A2-8 (Fig. 2c), the frequency of $GPC3_{144–152}$ peptide-specific CTLs was 39 of 5×10^5 PBMCs 1.5 months after the third vaccination, as determined by *ex vivo* ELISPOT assay, which were stimulated with $GPC3_{144–152}$ peptide *in vitro*. After 14 days, the population of $CD8^+$ GPC3 $Dextramer^+$ cells was 1.4% of all stimulated cells. We incubated $CD8^+$ T cells with T2 pulsed with $GPC3_{144–152}$, $HIV_{19–27}$ peptide, or HepG2. Approximately 2% and 18.7% of $CD8^+$ T cells mobilized CD107a in response to HepG2 and T2 pulsed with $GPC3_{144–152}$ peptide, respectively, but not in response to T2 pulsed with $HIV_{19–27}$ peptide. $CD107a^+$ $CD8^+$ cells that reacted against HepG2 were sorted to a single cell. The established clone was $CD8^+$ GPC3

Dextramer$^+$ CTLs (99.9%) which did not react with HIV Dextramer (Fig. 2c). These results indicate that GPC3$_{144–152}$ peptide-specific CTL clones were successfully established from PBMCs of patients injected with GPC3$_{144–152}$ peptide vaccine by three different methods. Moreover, the result that patient A2-8 CTL clone that reacted to HepG2 had GPC3$_{144–152}$ peptide specificity verified that GPC3$_{144–152}$ peptide was present naturally on HepG2.

Analysis of GPC3$_{144–152}$ peptide-specific avidity of three CTL clones. To further characterize the GPC3$_{144–152}$ peptide-specific avidity of the three CTL clones, we tested for the lysis of T2 cells pulsed with decreasing concentrations of GPC3$_{144–152}$ or HIV$_{19–27}$ peptide ranging from 10^{-6} to 10^{-14} M. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone. The recognition efficiencies of patient A2-9, A2-14, and A2-8 clones were 10^{-10}, 10^{-10}, and 10^{-11} M, respectively (Fig. 3). These CTL clones did not react against T2 cells pulsed with HIV$_{19–27}$ peptide. These results indicate that the established clones were GPC3$_{144–152}$ peptide-specific and high avidity CTLs.

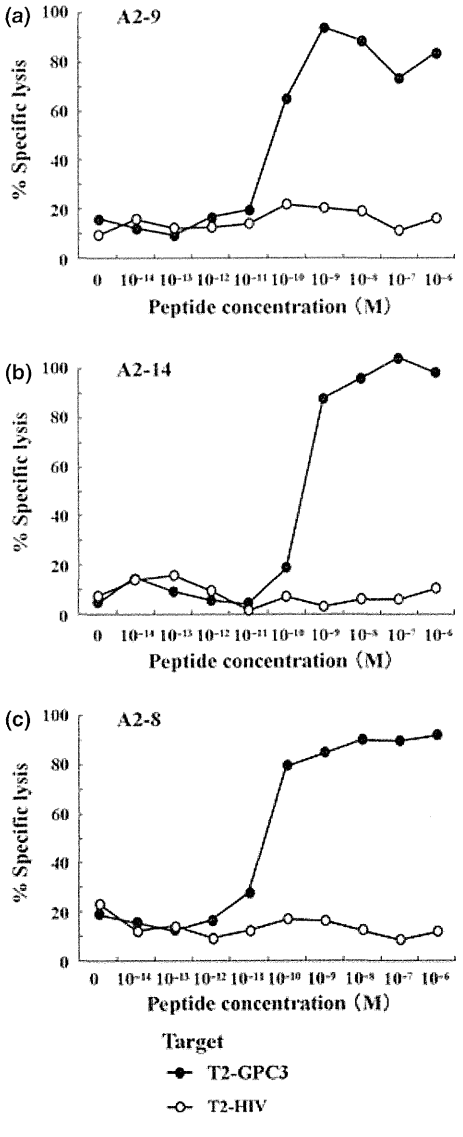

Fig. 3. Analysis of the GPC3$_{144–152}$ peptide specific avidity of the three CTL clones. The established CTL clones were tested for their avidities using various concentrations of GPC3$_{144–152}$ (●) or HIV$_{19–27}$ (○) peptide-loaded T2 targets. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone. Effector/target ratio = 10. The recognition efficiencies of patient A2-9 (a), A2-14 (b), and A2-8 (c) CTL clones were 10^{-10}, 10^{-10}, and 10^{-11} M, respectively.

Reactivity of three CTL clones against cancer cell lines. We analyzed the IFN-γ production and cytotoxicity of the established CTL clones against cancer cell lines expressing HLA-A*02:01 and GPC3. We used SK-Hep-1 (GPC3$^-$, HLA-A*02:01$^+$) and a human GPC3 gene transfectant, SK-Hep-1/hGPC3 (GPC3$^+$, HLA-A*02:01$^+$), as target cells. Production of IFN-γ in the three CTL clones was detected against SK-Hep-1/hGPC3, but not against SK-Hep-1 (Fig. 4a). Furthermore, these CTL clones showed specific cytotoxicity against SK-Hep-1/hGPC3 and HepG2 (GPC3$^+$, HLA-A*02:01$^+$), but not against SK-Hep-1 and SW620 (GPC3$^-$, HLA-A*02:01$^+$) (Fig. 4b). These results indicate that all three CTL clones show cytotoxicity and the ability to produce IFN-γ against HLA-A*02:01$^+$ GPC3$^+$ HCC cell lines. Next, we examined whether these CTL clones respond to cancer cells weakly expressing GPC3. We used human melanoma cell line 526mel (GPC3$^+$, HLA-A*02:01$^+$) as a target cell that expresses GPC3 mRNA and protein at a lower level than the HCC cell lines (data not shown). Production of IFN-γ in patient A2-8 CTL clone (recognition efficiency: 10^{-11} M) were clearly detected against 526mel, whereas patient A2-9 CTL clone (recognition efficiency: 10^{-10} M) showed weak response to 526mel (Fig. 4c). Similarly, patient A2-8 CTL clone showed specific cytotoxicity against 526mel, whereas patient A2-9 CTL clone failed to lyse 526mel (Fig. 4d). These results suggest that higher avidity is essential to react to cancer cells weakly expressing GPC3.

Analysis of HLA-A2 and GPC3 restriction. In a cold target inhibition assay, cytotoxicity against SK-Hep-1/hGPC3 of patient A2-9 clone was suppressed by the addition of GPC3$_{144–152}$ peptide-pulsed T2 cells but not by the addition of HIV$_{19–27}$ peptide-pulsed T2 cells (Fig. 5a). In an HLA blocking experiment, the IFN-γ production of patient A2-9 CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb as compared with that by IgG2a or IgG2b isotype control ($P < 0.05$) (Fig. 5b). Similarly, the cytotoxicity against SK-Hep-1/hGPC3 of patient A2-9 clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb compared with that by IgG2a and IgG2b isotype control ($P < 0.05$) (Fig. 5c). These results clearly indicate that the CTL clone recognized SK-Hep-1/hGPC3 in an HLA-A2-restricted manner.

Next, to ascertain the GPC3 antigen-specific response of a CTL clone, we examined GPC3 knockdown using siRNA on the GPC3$^+$ HepG2 cell line. Representative data are shown in Figure 5(d–f). The GPC3 expression of HepG2 was clearly decreased by GPC3 siRNA on RT-PCR (Fig. 5d). Specifically, the GPC3 expression of HepG2 was decreased from 24 to 72 h following treatment with GPC3 siRNA on Western blot (Fig. 5e). We examined the IFN-γ production of patient A2-9 CTL clone against HepG2 treated with GPC3 siRNA. The IFN-γ production of the CTL clone was significantly decreased by GPC3 siRNA ($P < 0.05$) (Fig. 5f). These results indicate that HLA-A2-restricted GPC3$_{144–152}$ peptide can be processed naturally by cancer cells, and the peptides in the context of HLA-A2 can be expressed on the cell surface of cancer cells in order to be recognized by a GPC3$_{144–152}$ peptide-specific CTL clone.

Discussion

Salgaller *et al.*[17] failed to detect dose dependency between 1 and 10 mg in terms of the capacity of gp100 peptide to enhance immunogenicity in humans. Previously, we reported that the peptide emulsified with incomplete Freund's adjuvant is stable, although the peptide is easily degraded in serum.[16] In this study, as with our previous report using a mouse model,[16] we

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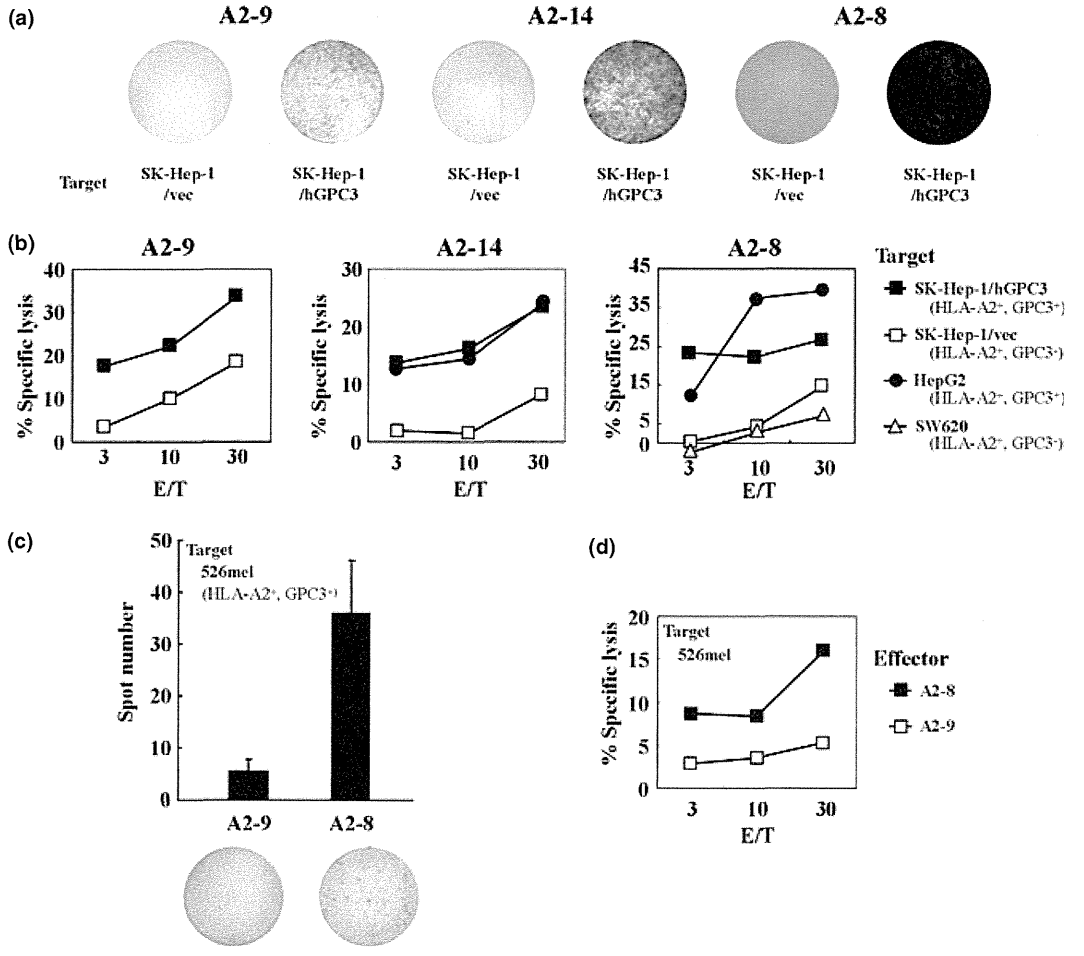

Fig. 4. Reactivity of three CTL clones against cancer cell lines. (a) γ-Interferon enzyme-linked immunospot assay of established CTL clones against SK-Hep-1/hGPC3 and SK-Hep-1/vec. Effector/target (E/T) ratio = 0.2. (b) Cytotoxic activities of the three CTL clones against SK-Hep-1/hGPC3 (■), SK-Hep-1/vec (□), HepG2 (●), or SW620 (Δ) analyzed by cytotoxicity assay. (c) γ-Interferon enzyme-linked immunospot assay of established CTL clones against 526mel. E/T ratio = 0.2. (d) Cytotoxic activities of patient A2-8 (■) and A2-9 (□) CTL clone against 526mel analyzed by cytotoxicity assay.

found that the effect of $GPC3_{144–152}$ peptide emulsified with incomplete Freund's adjuvant between 0.3 and 30 mg, to induce specific CTLs, was dose-dependent.

$GPC3_{144–152}$ (FVGEFFTDV) peptide was previously identified as an HLA-A*02:01-restricted peptide.[15] Moreover, we confirmed by binding assay that the peptide could also bind HLA-A*02:06 and HLA-A*02:07 molecules (data not shown). Therefore, we carried out a clinical trial for three types of HLA-A2 patient. Indeed, similar to HLA-A*02:01 patients, $GPC3_{144–152}$ peptide-specific CTLs increased after vaccination in both HLA-A*02:06 and HLA-A*02:07 patients (Fig. 1b). These findings suggest that $GPC3_{144–152}$ peptide is useful for not only HLA-A*02:01 patients but also HLA-A*02:06 and HLA-A*02:07 patients.

Notably, previous reports have shown that vaccination with synthetic peptides occassionally induced ineffective CTL responses due to various underlying mechanisms.[4–9] A possible mechanism is that responding T cells may have a very low affinity such that they recognize only target cells pulsed with high concentrations of the peptide and not tumor cells expressing the relevant epitopes at lower copy numbers. Alternatively, some antigen epitopes were not expressed on the surface of tumor cells.[18,19] When evaluating T-cell response to peptide vaccines, it is important to confirm that responding CTLs lyse human cancer cells. In the present study, although CTL clones established by Dextramer assay could react to HLA-A*02:01$^+$ GPC3$^+$ HCC cell lines, these clones failed to react to the HLA-A*02:01$^+$ GPC3$^+$ melanoma cell line 526mel expressing GPC3 mRNA and protein at a lower level than the HCC cell lines. Therefore, we attempted to establish CTL clones that are more tumor-reactive and with higher avidity than CTL clones established by Dextramer assay. Rubio *et al.*[20] showed that the surface mobilization of CD107a was useful for identifying and isolating functional tumor-reactive T cells with high recognition efficiency directly from PBMCs of cancer patients after vaccination. In the present study, the CTL clone showing the highest avidity (10^{-11} M) and tumor reactivity was established by CD107a mobilization assay. Moreover, this clone could also react to 526mel.

For patients with metastatic melanoma, adoptive cell therapy has emerged as the most effective treatment.[21,22] However, tumor-infiltrating lymphocytes with high avidity for tumor antigens can only be generated from some patients with melanoma.[21] Recent studies have shown that genes encoding T-cell receptors (TCRs) can be isolated from high avidity T cells that recognize cancer antigens, and retroviral or lentiviral vectors can be used to redirect lymphocyte specificity to these cancer antigens.[23–26] In the present study, we were able to successfully establish some high avidity CTL clones. We analyzed the TCR β-chain variable region gene families of these clones by

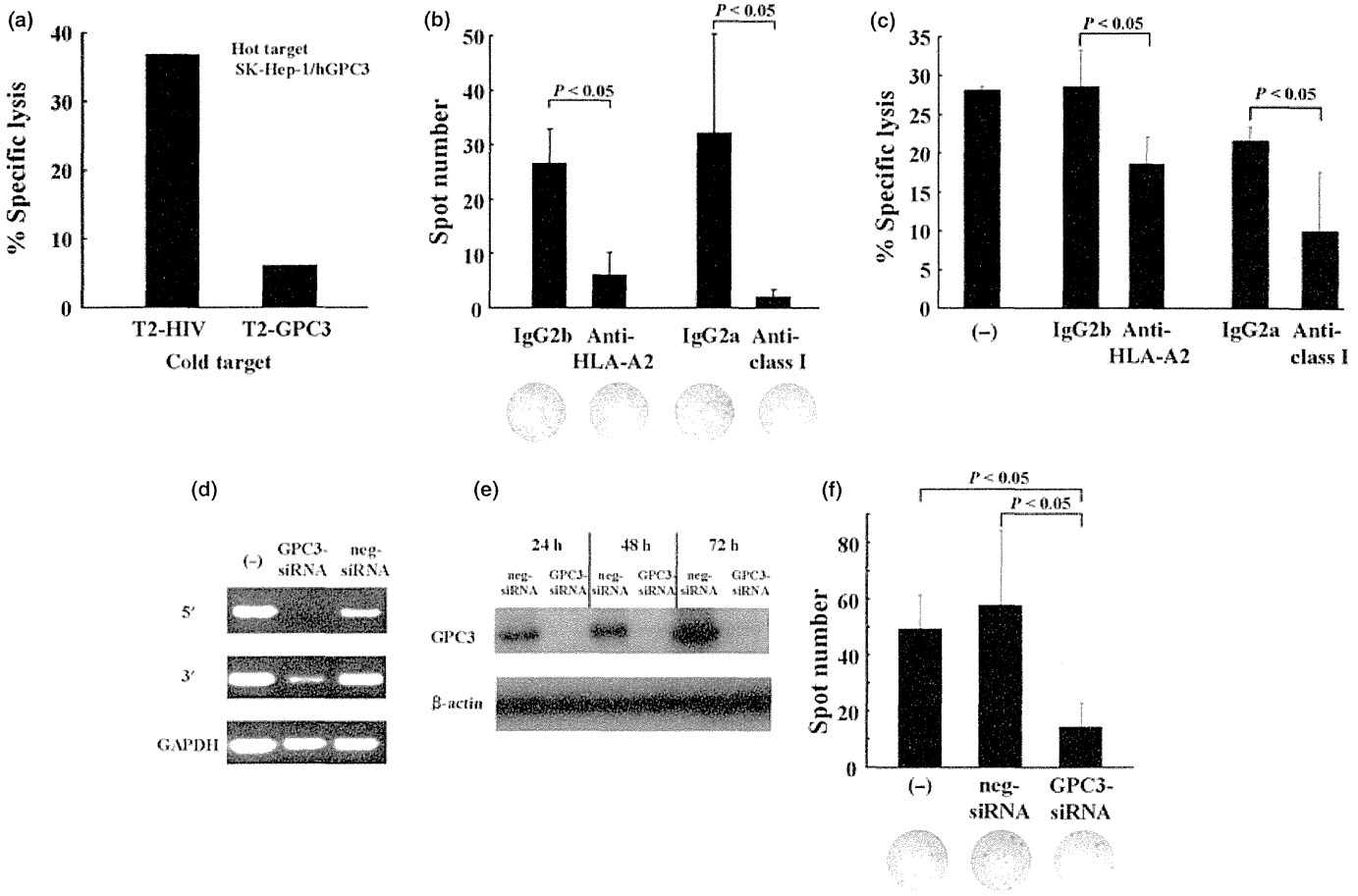

Fig. 5. Analysis of HLA-A2 and glypican-3 (GPC3) restriction. (a) Cold target inhibition assay of patient A2-9 CTL clone against SK-Hep-1/hGPC3. Effector/target (E/T) ratio = 30. T2 was prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide, then used as cold target cells. Cold/hot target ratio = 10. The cytotoxicity of the CTL clone was inhibited by T2 pulsed with $GPC3_{144-152}$ peptide but not by T2 pulsed with HIV_{19-27} peptide. (b) Inhibition of interferon (IFN)-γ production by anti-HLA class I mAb and anti-HLA A2 mAb. SK-Hep-1/hGPC3 used as target cells. E/T ratio = 0.02. The IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb as compared with that by IgG2a and IgG2b isotype control ($P < 0.05$). Data are expressed as the mean ± SD. (c) Inhibition of cytotoxicity by anti-HLA class I mAb and anti-HLA A2 mAb. SK-Hep-1/hGPC3 used as target cells. E/T ratio = 30. The cytotoxicity of the CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb compared with that by IgG2a and IgG2b isotype control ($P < 0.05$). (d) The GPC3 expression on HepG2 treated with GPC3-siRNA or negative (neg)-siRNA for 24 h as determined by RT-PCR. (e) The GPC3 expression on HepG2 treated with GPC3-siRNA or neg-siRNA from 24 to 72 h as determined by Western blot analysis. The GPC3 expression of HepG2 was decreased from 24 to 72 h after treatment with GPC3 siRNA. (f) The IFN-γ production of the CTL clone against HepG2 treated with GPC3 siRNA. E/T ratio = 0.02. The IFN-γ production of the CTL clone was decreased by GPC3 siRNA ($P < 0.05$). Data are expressed as the mean ± SD.

RT-PCR and carried out gene sequencing (data not shown). These clones had different TCR genes. Our results raise the possibility that these clones might be applicable to adoptive cell therapy for a large number of HCC patients.

In conclusion, we proved in this study the dose-dependent effects of highly immunogenic $GPC3_{144-152}$ peptide. Furthermore, we provided substantial evidence that CTLs showing not only high avidity but also natural antigen-specific killing activity against HCC cells could be induced in HCC patients by peptide vaccine.

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Disclosure Statement

The authors have no conflict of interest.

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Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma

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Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones from hepatocellular carcinoma patients after $GPC3_{144-152}$ peptide vaccination. The present study was designed to evaluate the tumor reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian clear cell carcinoma (CCC) cell lines. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines on interferon (IFN)-γ enzyme-linked immunospot assay and showed cytotoxicity against KOC-7c cells. The CTL clone recognized naturally processed GPC3-derived peptide on ovarian CCC cells in a HLA class I-restricted manner. Moreover, we confirmed that the level of GPC3 expression was responsible for CTL recognition and that subtoxic-dose chemotherapy made tumor cells more susceptible to the cytotoxic effect of CTL. Thus, it might be possible to treat ovarian CCC patients by combining chemotherapy with immunotherapy. Our data suggest that GPC3 could be an effective target for immunotherapy against ovarian CCC. (*Cancer Sci* 2011; 102: 1622–1629)

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Cytoreductive surgery and systemic combination chemotherapy with a platinum drug and a taxane represent the standard of care for EOC patients. Ovarian clear cell carcinoma (CCC) is the second most frequent subtype of EOC in Japan, although CCC represents 8–10% of all EOC in the United States.[1,2] Compared with other EOC subtypes, ovarian CCC is associated with a poorer prognosis and increased chemoresistance.[1,3] More efficient conventional therapies and novel strategies for effectively treating ovarian CCC are required.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via the glycosylphosphatidylinositol (GPI) anchor.[4] It is known as an oncofetal antigen specifically overexpressed in hepatocellular carcinoma (HCC).[5] Previous studies have shown that GPC3 was also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian CCC and lung squamous cell carcinoma.[6–10]

We previously identified the HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive cytotoxic T cells (CTL).[11] Recently, HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones were established from HCC patients after $GPC3_{144-152}$ peptide vaccination in our laboratory.[12] Although CTL reactivity against HCC cell lines was analyzed using these CTL clones, other GPC3-positive tumor cell lines have not been studied. Therefore, we examined the reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian CCC cell lines, and whether subtoxic-dose chemotherapy sensitizes ovarian CCC cells to lysis of $GPC3_{144-152}$ peptide-specific CTL.

Materials and Methods

$GPC3_{144-152}$ peptide-specific CTL clone and cell lines. We established the HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone from the PBMC of HCC patients vaccinated with $GPC3_{144-152}$ (FVGEFFTDV) peptide by single-cell sorting using CD107a antibody. The established CTL clone was tested for avidity by using $GPC3_{144-152}$ peptide-pulsed T2 targets with a range of peptide concentrations, starting at 10^{-6} M and decreasing by log steps to 10^{-14} M. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the avidity of the CTL clone and was rounded to the nearest log. This CTL clone had high avidity CTL (10^{-11} M) and could recognize HCC cell lines expressing GPC3 in a HLA-class-I-restricted manner.[12] Two human ovarian CCC cell lines, KOC-7c (HLA-A*0201/A*3101) and TOV-21G (HLA-A*1101/A*2601), and two human HCC cell lines, HepG2 (HLA-A*0201/A*2402) and SK-Hep-1 (HLA-A*0201/A*2402), were used in the present study. They were conserved in our laboratory. TOV-21G.A2 acquires expression of HLA-A2 following transfection with an HLA-A2 expression plasmid.[13] TOV-21G.A24 was similarly transfected with an HLA-A24 expression plasmid. SK-Hep-1.hG acquires expression of human GPC3 following transfection with a human GPC3 expression plasmid. SK-Hep-1.vec cell line transfected with an empty vector was used as a control. To study the effect of silencing GPC3, KOC-7c GPC3-shRNA and Neg-shRNA (control shRNA) were established by short hairpin RNA knockdown technology as described previously.[14] These cells were maintained in RPMI 1640 or DMEM medium (Sigma, St Louis, MO, USA) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA preparation and quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. GPC3 gene expression levels were analyzed by qRT-PCR assays using the following primers generated according to the indicated reference sequences: sense, 5′-GAGCCAGTGGTCAGTCAAAT-3′ and antisense, 5′-CTTCATCATCACCGCAGTC-3′. Amplification reactions were carried out in 96-well plates in 25 μL reaction volume using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All reactions were

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performed in technical triplicate using an ABI 7500 Fast Real-Time PCR System. Relative expression of the GPC3 gene to the endogenous control gene, β-actin, was calculated using the comparative C_T method. β-actin qRT-PCR primer sequences were: sense, 5′-TCCATCATGAAGTGTGACGT-3′ and antisense, 5′-GAGCAATGATCTTGATCTTCAT-3′.

Flow cytometry analysis and cell sorting. Flow cytometry (FCM) was performed to quantify the expression of GPC3 and Fas on the cell surface using the following antibodies: primary anti-GPC3 (clone 1G12; BioMosaics, Burlington, VT, USA); Alexa Fluor 488 conjugated second Ab (Invitrogen); phycoerythrin (PE)-conjugated anti-Fas (clone DX2; BioLegend, San Diego, CA, USA); FITC-conjugated anti-HLA-A2 (clone BB7.2; MBL, Nagoya, Japan); and FITC-conjugated mouse IgG2b isotype control (clone 3D12; MBL).

The FCM data was acquired using the FACSCanto II system (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Mean fluorescence intensity (MFI) of GPC3 staining was calculated as follows: MFI ratio = MFI with the anti-GPC3 Ab/MFI with the secondary Ab. MFI of HLA-A2 staining was similarly calculated (MFI ratio = MFI with the anti-HLA-A2 Ab/MFI with isotype control Ab).

Cell sorting was performed using the FACSAria II cell sorter (BD Biosciences) to isolate $GPC3^+$ and $GPC3^-$ cells from KOC-7c cells. We purified KOC-7c GPC3 high or low cells with the top or bottom 10% of GPC3 expression, respectively.

Response of $GPC3_{144\text{-}152}$ peptide-specific CTL clone against cancer cell lines. $GPC3_{144\text{-}152}$ peptide-specific CTL clone cells were co-cultured with each cancer cell line as target cells at the indicated effector/target (E/T) ratio and cytotoxicity assay or IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed. Blocking of HLA class I was done as follows. Before coculturing the CTL clone with a cancer cell line in an assay, the target cancer cells were incubated for 1 h with anti-HLA class I mAb (clone W6/32; BioLegend), or isotype control IgG2a mAb, and then the effects of Ab on CTL clone activity was examined.

IFN-γ ELISPOT analysis. ELISPOT assay for detecting antigen-specific IFN-γ-producing T cells was performed using the ELISPOT kit (BD Biosciences). The spots were automatically counted and analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. The cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech). The CTL clone was used for effector cells. Target cells were labeled in calcein-AM solution for 30 min at 37°C. The labeled cells were then co-cultured with effector cells for 4–6 h. Fluorescence intensity was measured before and after the 4–6 h culture, and specific cytotoxic activity was calculated as previously described.[12]

Cold inhibition assay. Calcein AM-labeled target cells were cultured with effector cells in a 96-well plate with cold target cells. T2 target cells, which were prepulsed with either $HIV_{19\text{-}27}$ peptide or $GPC3_{144\text{-}152}$ peptide, were used as cold target cells.

CD107a degranulation assay. $GPC3_{144\text{-}152}$ peptide-specific CTL clone cells were incubated with cancer cell lines at a 2:1 ratio for 4 h at 37°C. APC-conjugated CD107a-specific mAb (clone H4A3; BD Biosciences) were present during the incubation period; after incubation, cells were stained with additional PE-conjugated anti-CD8 mAb (clone HIT8a; BioLegend) and analyzed by FCM.

Growth inhibition assay. Growth inhibition was evaluated by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) colorimetric assay using a Cell Counting Kit (Dojindo, Kumamoto, Japan). Cells (5×10^3) were seeded into 96-well plates in 100 μL of culture medium for 24 h prior to drug exposure, and then treated with various concentrations of paclitaxel (PTX) or cisplatin (CDDP) for 18 or 48 h. Cell viability was determined colorimetrically by optical density at 450 nm wavelength using a microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell survival for each drug concentration was calculated as: (absorbance of test wells/absorbance of control wells) × 100.

Apoptosis analysis. The Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) was used to determine apoptosis after treatment with PTX or CDDP. After treatment with the chemodrug, floating and adhering cells were collected via trypsinization and centrifuged. The supernatant was removed and resuspended in 500 μL of binding buffer to which 5 μL of Annexin-V-FITC and propidium iodido (PI) was added. The cells were incubated at room temperature for 5 min in the dark and assessed by FCM.

Statistical analysis. Univariate regression analysis was used to evaluate the correlation between GPC3 expression and GPC3-specific CTL recognition. Mann–Whitney *U*-test and Kruskal–Wallis test followed by Scheffe's *post hoc* test were used to detect differences between groups. For all statistical tests, differences were considered significant at $P < 0.05$.

Results

HLA-A2-restricted $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines. To ascertain whether the HLA-A2-restricted $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines expressing HLA-A2 and GPC3, we first evaluated the expression of GPC3 on cancer cell lines. We used KOC-7c and HLA-A0201 gene stable transfectant TOV-21G.A2 and two human HCC cell lines for the target cells. As positive controls, we used two HCC cell lines. SK-Hep-1.hG cells were an established stable GPC3-expressing cell line. As we performed qRT-PCR and FCM of GPC3 in these cell lines, GPC3 expression in ovarian CCC cell lines was less than that in HCC cell lines. Representative data of relative mRNA expression (ratio to KOC-7c) and MFI ratio are shown (Fig. 1A). The CTL response generally correlates with the numbers and density of MHC/antigen peptide complex on the target cells. Accordingly, we also evaluated HLA-A2 expression on the cell surface in cancer cell lines with FCM analysis (Fig. 1B). IFN-γ production of the CTL clone was detected against two ovarian CCC cell lines (Fig. 1C). In Figure 1C, we used TOV-21G.A24 as a negative control. Furthermore, we determined whether efficient $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognition was correlated with GPC3 expression levels. We found that CTL clone recognition was correlated with the relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines ($r^2 = 0.995$ and 0.935, respectively) (Fig. 1D,E). In addition, we also analyzed whether CTL reactivity is correlated with not only GPC3 expression but also the expression of HLA-A2. The correlation between HLA-A2 expression levels on FCM analysis and CTL clone recognition (IFN-γ production or CD107a degranulation) was insufficient in the cell lines (data not shown). Although HLA-A2 expression on the cell surface in TOV-21G.A2 was moderately low, that in three other cell lines was sufficient on FCM analysis. TOV-21G.A2 cells have low expression of not only HLA-A2 but also GPC3. Therefore the GPC3 expression level is more important than the HLA-A2 expression level on $GPC3_{144\text{-}152}$ peptide-specific CTL clone reactivity.

$GPC3_{144\text{-}152}$ peptide-specific CTL clone lyses ovarian CCC cell lines. We detected GPC3-specific CTL responses by a CD107a degranulation assay. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively, approximately 1.8- and 3.4-fold increases compared with the SK-Hep-1.vec as a negative control (Fig. 2A). CD107a degranulation was also correlated with the

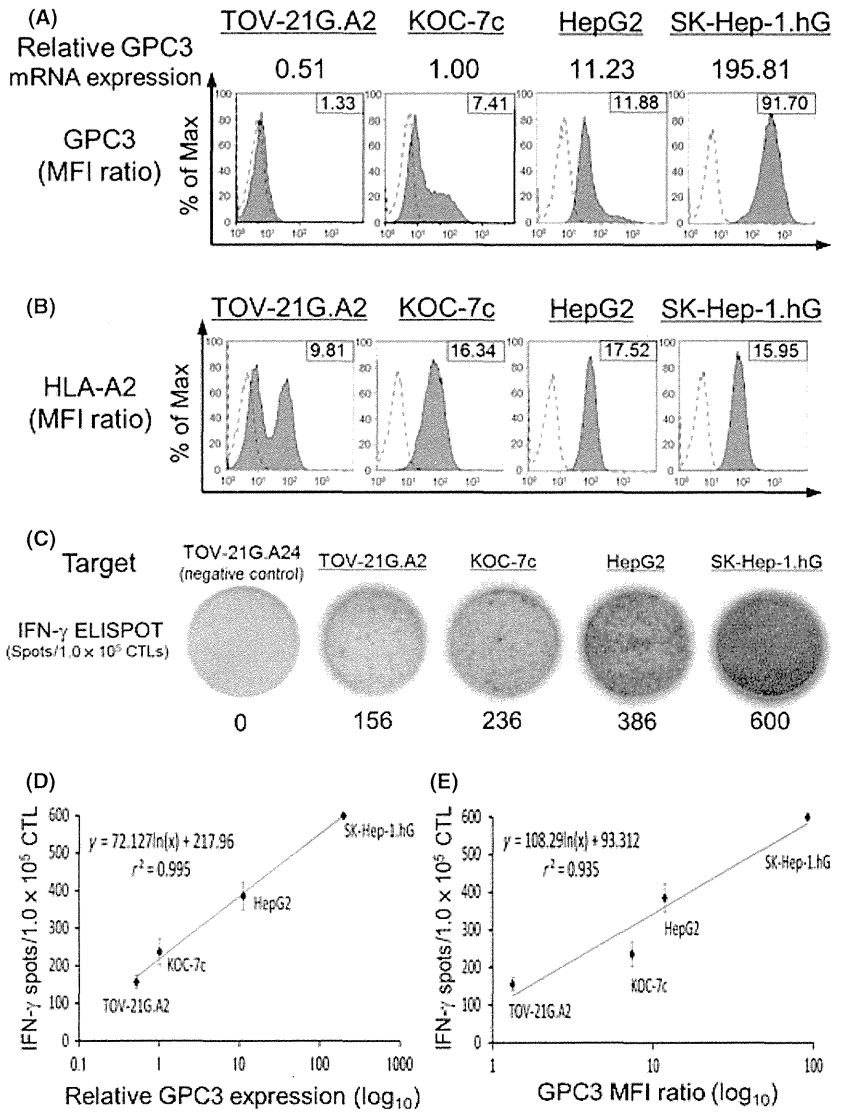

Fig. 1. HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian clear cell carcinoma (CCC) cell lines. (A) Expression of GPC3 on cancer cell lines. We used two human ovarian CCC cell lines (TOV-21G.A2 and KOC-7c) and two human HCC cell lines. We performed qRT-PCR and flow cytometry analysis (dashed line, secondary Ab stained control; gray-filled area, GPC3 staining). Numbers in the histograms correspond to the ratio of mean fluorescence intensity (MFI) of GPC3 staining, calculated as: MFI ratio = (MFI with the anti-GPC3 Ab)/(MFI with the secondary Ab). Representative data of relative GPC3 mRNA expression (ratio to KOC-7c) and GPC3 MFI ratio are shown. GPC3 expression in ovarian CCC cell lines was less than in HCC cell lines. (B) Expression of HLA-A2 on cancer cell lines. Numbers in histograms correspond to the ratio of MFI of HLA-A2 staining, calculated as: MFI ratio = (MFI with the anti-HLA-A2 Ab)/(MFI with isotype control Ab). (C) Representative results of IFN-γ ELISPOT analysis are shown. Effector/target ratio = 2. TOV-21G.A24 cells were used as a negative control. (D) IFN-γ production of a $GPC3_{144-152}$ peptide-specific CTL clone was correlated with relative GPC3 mRNA expression (r^2 = 0.995). (E) Similarly, $GPC3_{144-152}$ peptide-specific CTL clone recognition was correlated with the GPC3 MFI ratio (r^2 = 0.935).

relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines (r^2 = 0.978 and 0.865, respectively) (Fig. 2B). The $GPC3_{144-152}$ peptide-specific CTL clone was further tested for its capacity to kill ovarian CCC cell lines, by a calcein-AM-based cytotoxicity assay. SK-Hep-1.vec cells were used for a negative control. The CTL clone displayed mild, but clear, specific cytotoxicity against KOC-7c cells (Fig. 2C). However, GPC3-specific cytotoxicity was insufficient against TOV-21G.A2 cells compared with TOV-21G.A24 cells (data not shown). In both ovarian CCC cell lines, Fas expression on the cell surface was sufficiently similarly to that of the HCC cell lines on FCM analysis (Fig. 2D).

HLA class I specificity was confirmed by the blockade of reactivity against ovarian CCC cell line KOC-7c. HLA class I-restricted activity was demonstrated by blocking of IFN-γ release and lysis of the $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c after pretreatment with a HLA class I-specific mAb (W6/32) or mouse IgG2a isotype control, respectively, for 1 h. This reactivity could be inhibited by anti-HLA class I mAb but not by isotype control (Fig. 3). These results clearly indicate that the CTL clone recognized KOC-7c in a HLA class I-restricted manner.

Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. To verify the GPC3 antigen-specific response of the CTL clone against ovarian CCC cell lines, we examined GPC3 knockdown on the GPC3-positive cell line KOC-7c. KOC-7c GPC3-shRNA was established using shRNA knockdown technology. The GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. We examined the IFN-γ production and

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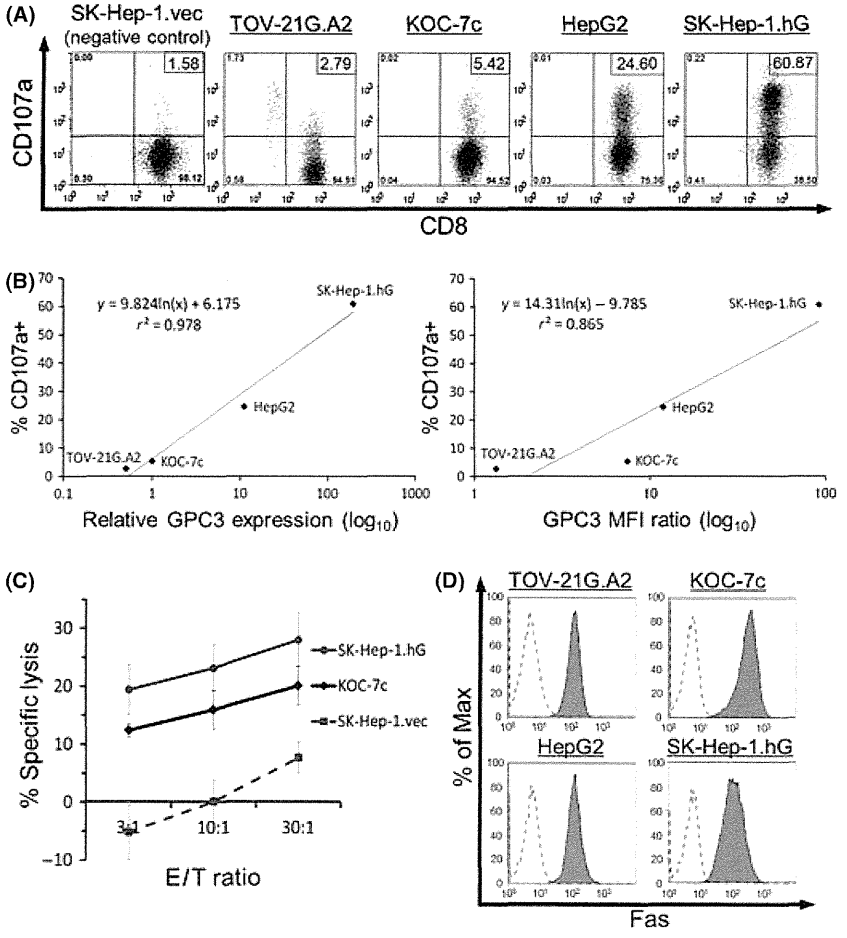

Fig. 2. $GPC3_{144-152}$ peptide-specific CTL clone lyses ovarian clear cell carcinoma (CCC) cell lines. (A) CD107a degranulation assay. Representative data are shown. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively. (B) CD107a degranulation was correlated with relative GPC3 mRNA expression and GPC3 mean fluorescence intensity (MFI) ratio in cell lines (r^2 = 0.978 and 0.865, respectively). (C) Cytotoxicity (4 h) assay was performed at three effector/target ratios. We used SK-Hep-1.hG as a positive control. SK-Hep-1.vec cells were used as a negative control. The CTL clone showed specific cytotoxicity against KOC-7c cells. Data represent the mean ± SD. (D) Flow cytometry analysis of Fas expression on cancer cell lines. In all cell lines, Fas expression was sufficient (dashed line, unlabelled control; gray-filled area, PE-Fas staining).

lysis of the CTL clone against KOC-7c GPC3-shRNA and KOC-7c GPC3 Neg-shRNA cells. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$) (Fig. 4A). GPC3-specific cytotoxicity was reduced against KOC-7c GPC3-shRNA cells compared with KOC-7c Neg-shRNA cells (Fig. 4B). These results indicate that HLA-A2-restricted $GPC3_{144-152}$ peptide could be processed naturally by ovarian CCC cells, and the peptides in the context of HLA-A2 could be expressed on the surface of ovarian CCC cells.

Level of GPC3 expression on the cell surface is related to $GPC3_{144-152}$ peptide-specific CTL clone recognition. To confirm that the level of GPC3 expression on the cell surface is responsible for CTL recognition, KOC-7c GPC3 high and low cells were sorted by FACSAria II (Fig. 5A). As shown in Figure 5B, KOC-7c GPC3 high cells expressed higher mRNA of GPC3 than GPC3 low cells. Figure 5C shows the IFN-γ release of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences in IFN-γ production between the three populations ($P < 0.001$). GPC3-specific cytotoxicity was increased against KOC-7c GPC3 high cells compared with GPC3 low cells in a cytotoxicity assay without cold target cells. In a cold target inhibition assay, cytotoxicity against KOC-7c GPC3 high cells was suppressed by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells, even though cytotoxicity against KOC-7c GPC3 low cells was not changed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide (Fig. 5D).

Chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of $GPC3_{144-152}$ peptide-specific CTL clone. Taxane plus platinum combination chemotherapy is generally considered to be the "gold standard" regimen for treatment of EOC. As PTX and CDDP have different mechanisms of action, we chose these two agents to investigate whether they sensitize ovarian CCC cells to GPC3-specific lysis. To evaluate the subtoxic dose of each drug, we assessed growth inhibition and apoptosis assays by FCM using Annexin V and PI staining. Growth-inhibitory effects were observed for treatment with either PTX or CDDP alone in a time- and dose-dependent manner. We calculated the 25% inhibitory concentration (IC25) of each drug as the minimum cytotoxic condition and regarded lower values as the subtoxic dose. The IC25 values of PTX and CDDP for 18 h were 22.8 ng/mL and 6.2 μg/mL, respectively (Fig. 6A). Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect, as determined by apoptosis assay. In other words, cell viability in untreated and PTX- and CDDP-treated groups of CTL clone or KOC-7c cells exceeded 95% in all cases (Fig. 6B). These

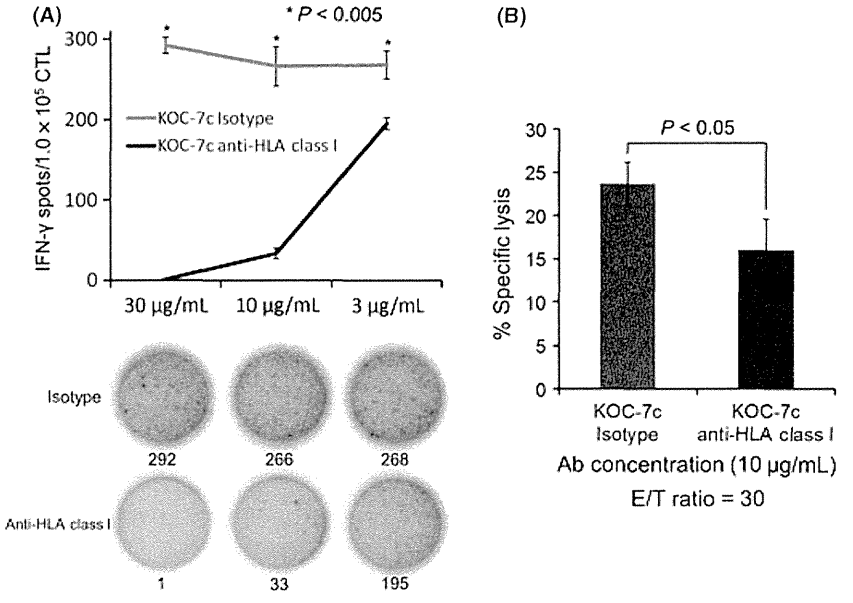

Fig. 3. Analysis of HLA class I restriction. (A) Inhibition of IFN-γ production by anti-HLA class I mAb. Effector/target ratio = 2. Data represent the mean ± SD of six wells. IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb compared with that by isotype control in a concentration-dependent manner (*$P < 0.005$). (B) Inhibition of cytotoxicity by anti-HLA class I mAb. Effector/target (E/T) ratio = 30. Ab concentration = 10 μg/mL. Data represent the mean ± SD from the 4 h cytotoxicity assay. Cytotoxicity could be inhibited by anti-HLA class I mAb but not by isotype control ($P < 0.05$).

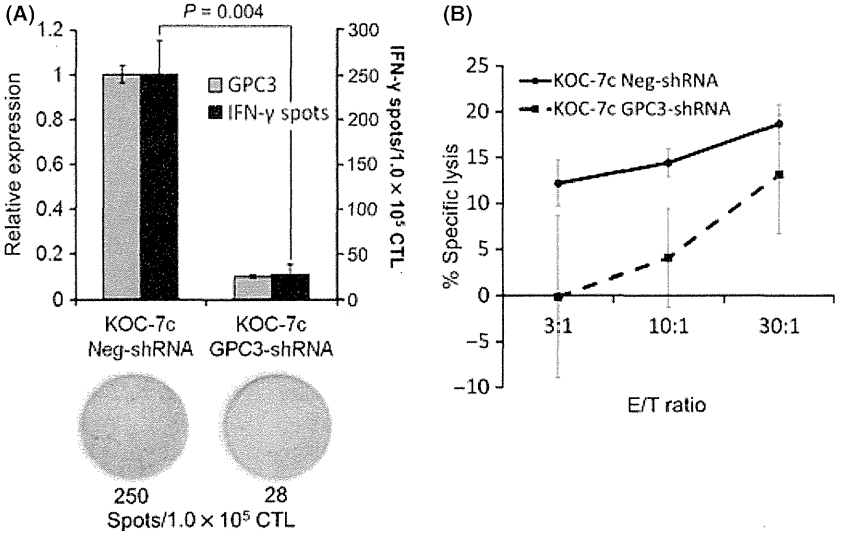

Fig. 4. Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. (A) GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$). Data represent the mean ± SD. Effector/target (E/T) ratio = 2. (B) KOC-7c GPC3-shRNA cells were less cytolytic than KOC-7c Neg-shRNA cells. Data represent the mean ± SD from the 4 h cytotoxicity assay.

conditions excluded direct cytoxic effects of the compounds and effects as a subtoxic dose. In contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity (basal levels of apoptosis >5%), and PTX (1 μg/mL) or CDDP (10 μg/mL) for 18 h induced substantial cell death (data not shown). KOC-7c cells were exposed to the subtoxic dose of each drug for 18 h and then examined by cytotoxicity assay. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (Fig. 6C). In all experiments, the level of spontaneous calcein release of target cells treated with chemotherapeutic agents was similar to that of untreated cells.

Discussion

Ovarian CCC has a poor prognosis due to low sensitivity to conventional chemotherapy.[1,3] To improve the prognosis, strategies are needed to efficiently kill all cancer cells by surgery and chemotherapy, as well as to stimulate the immune response to keep residual tumor cells in check. Thus, effective novel treatment strategies combined with surgery and chemotherapy are needed for treating ovarian CCC. Cancer vaccines are an attractive approach because of their low toxicity.

In previous studies, GPC3 was overexpressed in several malignant tumors, including ovarian CCC.[6–10] GPC3 is useful

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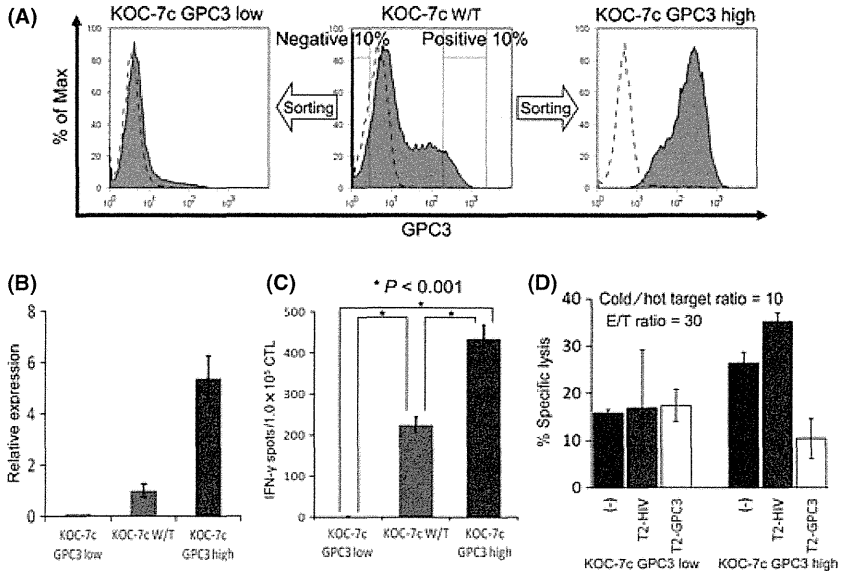

Fig. 5. The level of GPC3 expression on the cell surface is responsible for CTL recognition. (A) KOC-7c GPC3 high and GPC3 low cells were sorted as described in the Materials and Methods. (B) Relative GPC3 mRNA expression (ratio to KOC-7c wild type) is shown. Data represent the mean ± SD. (C) IFN-γ production of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences between the three populations (*$P < 0.001$). Mean ± SD of six wells is shown. (D) Cold target inhibition assay of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c GPC3 high and GPC3 low cells. Effector/target (E/T) ratio = 30. T2 was prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide and then used as cold target cells. Cold/hot target ratio = 10. Cytotoxicity of the CTL clone against KOC-7c GPC3 high cells was inhibited by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells. In contrast, cytotoxicity against the KOC-7c GPC3 low cells was not suppressed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide. Data represent the mean ± SD from the 4 h cytotoxicity assay.

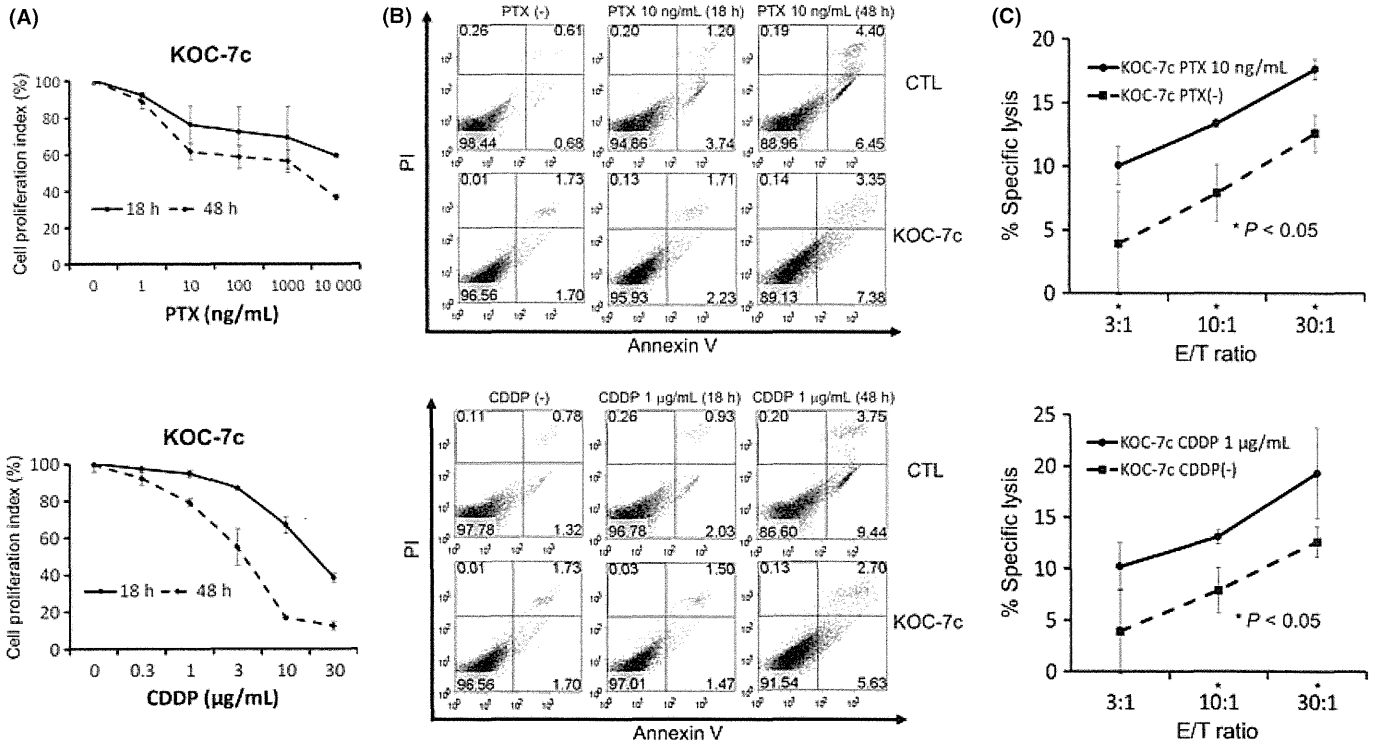

Fig. 6. Subtoxic-dose chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. We used two agents (paclitaxel [PTX] and cisplatin [CDDP]) to investigate whether they sensitize ovarian clear cell carcinoma (CCC) cells to GPC3-specific lysis. (A) Growth-inhibitory effects were observed for treatment with each drug alone in a time- and dose-dependent manner. Data represent the mean ± SD. (B) Apoptosis analysis by flow cytometry analysis. Representative data are shown. The numbers in each quadrant represent the percentage of cells in the quadrant. Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect. By contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity. (C) KOC-7c cells were pretreated with the subtoxic dose of each drug for 18 h and then a cytotoxicity assay (4 h) was performed. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (*$P < 0.05$). Data represent the mean ± SD.

as a novel biomarker and oncofetal antigen for immunotherapy.[15–22] However, association of ovarian CCC with CTL recognition has not been performed, hindering the selection of appropriate candidates for GPC3-specific immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones.[12] In the present study, we analyzed the IFN-γ production and cytotoxicity of an established CTL clone against ovarian CCC cell lines expressing HLA-A0201 and GPC3. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines, suggesting that ovarian CCC present endogenously processed $GPC3_{144-152}$ peptide. Even though the CTL clones recognized two ovarian CCC cell lines on the IFN-γ ELISPOT assay, they showed inefficient lysis against TOV-21G.A2 cells. This was not due to a low expression level of HLA-A2 molecules on the cell surface, because the tumor cells were lysed after being pulsed with the antigenic peptide (data not shown). We also confirmed that the level of antigen expression is important in GPC3-specific CTL recognition of malignant cells. Therefore, low-level expression of GPC3 on tumor cells might be insufficient for triggering CTL-mediated killing.

Recent clinical studies have reported high rates of objective clinical response when cancer vaccines are combined with chemotherapy in patients with various cancers.[23–27] To evaluate the feasibility of chemoimmunotherapy for ovarian CCC, we investigated the cytotoxic effect of subtoxic-dose PTX or CDDP combined with $GPC3_{144-152}$ peptide-specific CTL clone in the human ovarian CCC cell line KOC-7c. We found that chemotherapy made ovarian CCC cells more susceptible to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. Chemotherapeutic drugs generally suppress the immune function, and each drug has a different level of immune suppression. Therefore, combination therapy requires an optimal dose that does not suppress peptide-induced immune activation. Importantly, the synergistic cytotoxic effect remained when both CTL and tumor cells were pretreated with PTX or CDDP under identical conditions (data not shown). However, high-dose chemotherapy has been shown to be toxic and the synergistic effect increased slightly more compared with the subtoxic dose, therefore limiting its potential therapeutic usefulness *in vitro*. The mechanism of improvement in immunotherapy with chemotherapy remains unclear, but the two possible types of mechanism are: systemic factors and local tumor microenvironment factors. For example, possible systemic effects include the elimination of cells with immunosuppressive activity such as regulatory T cells[28] and myeloid-derived suppressor cells,[29] or improved cross-presentation of tumor antigens. Examples of possible local effects include the disruption of tumor stroma that results in improved penetration of CTL into the tumor site, increased permeability of tumor cells to CTL-derived granzymes via upregulation of mannose-6-phosphate (M6P) receptors on the surface of tumor cells,[30] increased expression of tumor-associated antigens by tumor cells or upregulation of Fas (and other death receptors) on tumor cells, or FasL on CTL, etc.[31,32] We performed experiments to address the change in permeability for GrzB and the expression of M6P receptors in KOC-7c cells pretreated with PTX or CDDP. However, both drugs had no significant effect on the expression of M6P receptors. Moreover, we could not confirm the mechanism through an increase in permeability to GrzB in CCC cell line KOC-7c cells. Paclitaxel is known to upregulate the expression of Fas on the surface of tumor cells, resulting in an increase in Fas–FasL interaction.[33] However, Fas expression was sufficient in ovarian CCC cell lines without chemotherapy, and both drugs had no significant effect on Fas expression. The threshold for Fas-induced apoptosis in ovarian CCC is high and/or Fas signaling in CCC is altered through unknown mechanisms. In addition, both drugs had no significant effect on GPC3 expression under subtoxic-dose conditions (data not shown).

In conclusion, the present study suggests that GPC3 could become an effective target for HLA-A2-restricted peptide vaccine therapy against ovarian CCC. Moreover, our data suggest the possibility of treating ovarian CCC patients by combining standard chemotherapy with relatively non-toxic and highly specific immunotherapy. We will clarify the mechanisms of this phenomenon in our next study.

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Disclosure Statement

The authors have no conflict of interest.

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