TNM 分類は国際対がん連合（UICC）が中心となって作成しているがんの進行度分類であり，Tつまり腫瘍の大きさや深さ，Nつまりリンパ節転移の状態，そしてMつまり遠隔転移の有無の3つの要素から決められている．初期の TNM 分類は乳癌，肺癌などを想定して作られているため，これを胃などの消化管にあてはめることには無理があった．すでに胃癌では，その予後を決定する最大の因子の1つは，がんの深達度でありその大きさでないことが明らかにされていたために，TNM 分類は直ちには我が国の胃癌分類として受け入れられることはなかった．また，すでに我が国ではリンパ流の精細な解析から，原発巣の局在ごとに領域リンパ節を同定して作られていた．この取扱い規約のリンパ節群分類に比較して，原発巣からの距離で分けた群分類は非科学的なものであった．『胃癌取扱い規約』は英文としても公開されたが，TNM 分類が世界の標準的病期分類として使用され続けてきた．その原因は2つあり，1つは日本のような広汎で精緻なリンパ節郭清が諸外国では不可能であったことである．もう1つは，切除標本から丁寧に時間をかけて，リンパ節を採取してマッピングを行い，それを病理医が検討するという我が国では当然の体制が，諸外国には存在していなかったことである．

TNM 分類は UICC が中心となって作成するが，現状ではその改訂にあたっては，米国の American Joint Committee on Cancer（AJCC）が大きな発言力を持っている．我が国からも TNM 分類の担当委員が討論に参加しているが，すべてのがん種に関する委員が網羅されているわけではない．例えば，胃癌のように日本が圧倒的に高いレベルの研究や診療を行っている分野でさえ，専門の委員が参加していない．TNM は 2010 年の改訂に向けて準備が進んでいたが，胃癌の分類については我が国はもちろん，国際的な胃癌研究機関である国際胃癌学会の意見も十分反映されていないことが 2008 年夏に明らかになった．国際胃癌学会の代表として佐野武博士と韓国の Han-Kwang Yang 教授が渡米し，日韓双方のデータを提示することにより，原案を適切に変更することができた．このような経験から，日本ばかりでなく国際胃癌学会の加盟諸国のデータをきちんと提示し，積極的に TNM 分類の作成にかかわる必要があることは明らかである．このような経緯が，2009 年のポーランドにおける国際胃癌学会の理

事会で説明され，今後国際胃癌学会としてのデータ作成を開始することが同意された．

ガイドラインの改訂点

我が国ではすでに『胃癌取り扱い規約』13 版から，TNM に沿った病期分類に変更しており，基本的な合意は得られている．しかし，『胃癌取扱い規約』14 版からはリンパ節転移（N）を解剖学的局在からではなく転移個数に変更するなど，大幅な変更が行われる可能性が高い．転移の局在同様，リンパ節転移の個数もその予後を決める重要な因子であることは，我が国のデータでも明らかであり，個数を採用すること自体は問題は少ない．しかし，解剖学的な転移の局在の記載は今まで同様に行われるべきであり，個数によって決定するからといって従来からの精緻な検討を放棄するべきではない．現在，『胃癌取り扱い規約』第 14 版は，表 1 のような最終案をもとに調整が進んでいる．

この新しい規約分類にガイドラインも対応して，表 2 のような案が作られて，これをもとに第 3 版の改訂作業が進められている．2009 年 3 月の日本胃癌学会総会では，『胃癌取扱い規約』と『胃癌治療ガイドライン』に関するコンセンサスミーティングが 3 時間にわたって開かれた．以下にそのときの結論と，ガイドライン改訂で問題点となっている主な項目について述べる．

早期胃癌の内視鏡治療（内視鏡的粘膜下層剥離術：ESD，内視鏡的粘膜切除術：EMR）に関しては，現在の適応は，分化型，2 cm 以下，M癌とされている．実際には高齢者や手術リスクの極めて高い例に対して，適応拡大が試みられている．例えば，サイズの小さな未分化型 M癌，分化型 SM1 癌の中には内視鏡治療の適応とされるものがあることを示すエビデンスも存在している（表 3）．しかし，その症例数が限られているために，第 2 版では適応を見送られた経緯がある．第 3 版ではそれを見直すほどのエビデンスが報告されていないため，当分は現在の適応は変わらないものと考えられる．内視鏡治療で重要なポイントは，一括切除可能で切除後にその深さや断端の病理学的に評価が適切に行えることである．これは，適応内の病変に関してももちろんであるが，拡大適応や診断的な目的で ESD を行う場合には特に

表1『胃癌取り扱い規約』(第14版案)

深達度(T)
- T0：癌がない
- T1：癌の浸潤が粘膜(T1a, M)または粘膜下組織(T1b, SM)にとどまるもの
- T2：癌の浸潤が粘膜下組織を超えているが，固有筋層(MP)にとどまるもの
- T3：癌の浸潤が固有筋層を超えているが，漿膜下組織(SS)にとどまるもの
- T4a：癌の浸潤が漿膜を破って遊離腹腔に露出しているもの(SE)
- T4b：癌の浸潤が周囲組織まで及ぶもの(SI)

リンパ節転移の程度(N)
- N0：領域リンパ節に転移を認めない
- N1：領域リンパ節に1～2個の転移を認める
- N2：領域リンパ節に3～6個の転移を認める
- N3：領域リンパ節に7個以上の転移を認める
  - N3a：7～15個の転移を認める
  - N3b：16個以上の転移を認める

	N0	N1 (1～2)	N2 (3～6)	N3 (7～)	Any N, M1
T1a (M), T1b (SM)	ⅠA	ⅠB	ⅡA	ⅡB	Ⅳ
T2 (MP)	ⅠB	ⅡA	ⅡB	ⅢA	
T3 (SS)	ⅡA	ⅡB	ⅢA	ⅢB	
T4a (SE)	ⅡB	ⅢA	ⅢB	ⅢC	
T4b (SI)	ⅢB	ⅢB	ⅢC	ⅢC	
Any T, M1	Ⅳ				

厳密に要求される点である．つまり，病理学的評価が困難な標本しか得られないような技術，あるいは病変であればESDを行うべきでないということである．内視鏡治療のデータベース化は紆余曲折を経て，日本胃癌学会の登録委員会で進行中である．予後をきちんと知るためには，一朝一夕では成立できない事業であり，学会が中心となって継続的に続けられるべきであろう．また，将来的には，適応拡大を行おうとする施設は，その全症例の登録を義務づけるべきであろう．コンセンサスミーティングでも，討論後の参加者によるアンサーパッドの結果では，現状の適応に満足せず適応拡大が必要とする意見が36％認められた(図1)．最近急速に普及してきた腹腔鏡下手術に関しては，前回のアンケート調査では日常診療にすべきとする意見が32％

表2　日常診療で推奨される進行度別治療法の適応（案）

	N0	N1	N2	N3	Any N, M1
T1a（M）	ⅠA ESD （分化型，2 cm 以下，UL（−）） 胃切除 D1 （上記以外）	ⅠB 胃切除 D1＋No. 8a, 9 （2.0 cm 以下） 胃切除 D2 （2.1 cm 以上）	ⅡA 胃切除 D2	ⅡB 胃切除 D2	Ⅳ
T1b（SM）	ⅠA 胃切除 D1（分化型，1.5 cm 以下） 胃切除 D1＋No. 8a, 9（上記以外）				
T2（MP）	ⅠB 胃切除 D2	ⅡA 胃切除 D2＋補助化療	ⅡB 胃切除 D2＋補助化療	ⅢA 胃切除 D2＋補助化療	
T3（SS）	ⅡA 胃切除 D2＋補助化療	ⅡB 胃切除 D2＋補助化療	ⅢA 胃切除 D2＋補助化療	ⅢB 胃切除 D2＋補助化療	
T4a（SE）	ⅡB 胃切除 D2＋補助化療	ⅢA 胃切除 D2＋補助化療	ⅢB 胃切除 D2＋補助化療	ⅢC 胃切除 D2＋補助化療	
T4b（SI）	ⅢB 胃切除 D2，合切＋補助化療	ⅢB 胃切除 D2，合切＋補助化療	ⅢC 胃切除 D2，合切＋補助化療	ⅢC 胃切除 D2，合切＋補助化療	
Any T, M1	Ⅳ 化学療法，緩和（姑息）手術，放射線治療，緩和医療				

表3　SM 癌でリンパ節転移のない条件（SM1，ly0，v0 の場合は 3 cm までは転移がない）（文献[1]より引用改変）

	分化型	リンパ節転移	%（95 %CI）
≦10 mm	28	0	0.0（0 ～ 12.3）
≦20 mm	59	0	0.0（0 ～ 6.1）
≦30 mm	58	0	0.0（0 ～ 6.2）
>31 mm	78	2	2.6（0 ～ 4.6）
	223	2	0.9（0 ～ 1.6）

CI：信頼区間　　国立がんセンター，癌研究会附属病院資料

図1　胃癌内視鏡治療の適応拡大について
（第 81 回日本胃癌学会総会コンセンサスミーティングより）

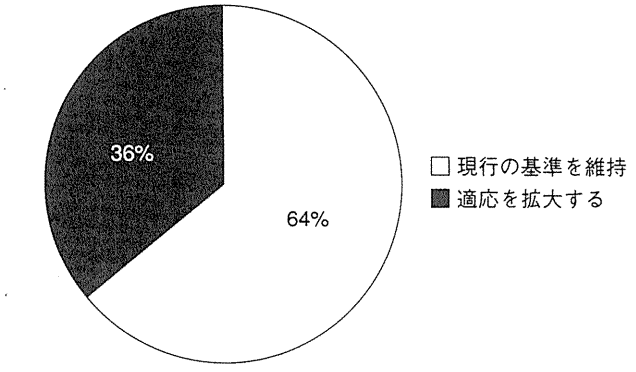
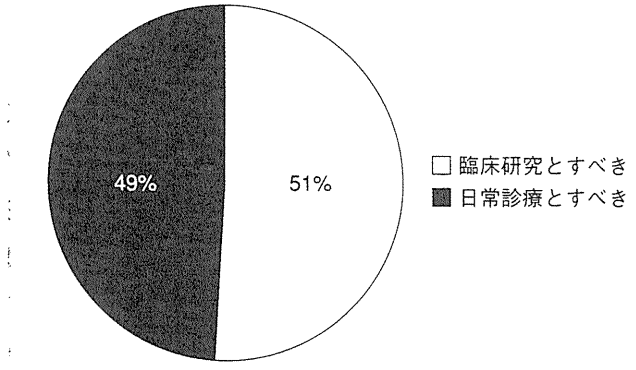


図2　腹腔鏡下胃切除について
（第 81 回日本胃癌学会総会コンセンサスミーティングより）



であったが，今回のアンサーパッドによる評価では，日常診療とする意見が 49％，研究的治療とする意見が 51％ であった．広く日常的な診療とするには時期尚早と思われる（図2）．

切除不能進行胃癌に対する化学療法レジメとしては速報版ですでに報告されているように，TS-1＋CDDP や TS-1 が標準的なものとして推奨されることになろう．また，ステージⅡ，Ⅲ胃癌の手術後の補助化学療法については，TS-1 の 1 年間の内服が推奨されることになる．しかし，このエビデンスは解剖学的局在に基づいた病期分類にしたがって作られているので，これらの取扱いについては整合性を図るために十分な検討が必要であろう．

ガイドラインの意義を見直す－おわりにかえて

ガイドラインについては 2 つの大きな流れに留意する必要がある．1 つは，国際化という流れであり，第 14 版の『胃癌取り扱い規約』には TNM 分類の第 7 版が大幅に取り入れられつつある．このような動きに対して，世界をリードする我が国の胃癌研究の基本である取扱い規約を，TNM に合わせるのは我が国の主張を後退させるものであると必ずしも賛成でないものもある．しかし，世界にはさまざまな医療環境が存在するのであり，今すぐ日本のような胃癌診療が実現す

ると考えるのは間違いであろう．日本が自国の状況と優位性にこだわらず，TNM 分類を取り入れることで，世界中のさまざまなレベルの国々と共通の言語で，情報を交換することには大いに意味がある．もちろん，ただ TNM を取り入れるだけではなく，日本は韓国などの胃癌先進国と共に，国際胃癌学会という国際的な組織をリードして精密なデータをそろえ，今後 TNM 分類をさらに正しい方向に改訂する必要がある．

もう1つの流れは，ガイドラインが普及するとともに明らかになってきた，ガイドラインの意義をめぐる誤解である．ガイドラインは規則でも法律でもないし，またマニュアルとも異なったもので，診療の大まかな流れを示したものに過ぎない．したがって，ガイドラインに100％の患者があてはまるものでもない．もし，100％あてはまるものを作ろうとすると，それは極めて大まかなものになってしまうし，反対に厳密に決め過ぎるとそれにあてはまる症例はごくわずかに限定されてしまう．どのように優れたガイドラインを作ろうとも，千差万別の臨床例をすべてカバーすることはできない．ところが，ガイドラインが100％守られるべきものと誤解，あるいは曲解しているものが見受けられる．例えば，もしガイドラインを100％遵守している施設があるとしたら，むしろそのような施設ではひたすらガイドラインを遵守することで，患者の個々の病状や背景を全く無視した医療を行っている可能性がある．このような病院が良い病院として高く評価されるようであれば，日本の医療レベルの低下は避けられない．このような考えのもとでは，ガイドラインは医療の多様性，不確実性を無視した画一的な医療を推し進める道具になってしまう．これは患者中心の医療とは全く対極にあるものである．『胃癌治療ガイドライン』が最初に作成されたときに，このような事態を予見して注意を喚起する意見があった．それが現実のものになろうとしたわけであり，今後も十分にこのような誤解や誤用がまかり通らないよう留意する必要がある．ガイドラインは必要なものであるが，諸刃の剣であり，使い方によっては有用にも，有害にもなることに留意したい．

山口俊晴

文　献

1) 日本胃癌学会編: 胃癌治療ガイドライン第2版. 金原出版株式会社, 東京, 2004.
2) 日本胃癌学会編: 胃がん治療ガイドラインの解説 一般用 2004 年 12 月改訂 (第2版) 胃がんの治療を理解しようとするすべての方のために. 金原出版株式会社, 東京, 2004.

厚生労働科学研究費補助金
(分担研究報告書)

がん診療ガイドラインの作成（新規・更新）と公開の維持およびその在り方に関する研究

平成21年度～平成23年度

(研究分担者　金子周一　金沢大学大学院恒常性制御学　教授)

研究要旨
肝癌診療ガイドラインの公開手順と維持については，改訂版でかなり改善されていたものの，公開方法や中心となる機関の設置など整備が必要である．ガイドライン更新の際には，外部評価や利用者アンケートなどの結果を十分反映して更新されるべきで，反映のためのよりよい仕組み作りも必要である．

A．研究目的

肝癌診療ガイドラインは，平成14-15年の厚生労働省ガイドライン支援事業により， 2005年初版が発表された．その後，組織を日本肝臓学会に移行し，2009年11月に改訂版が発表され，2011年9月より第3版の改訂作業が開始されている．

本研究では，このガイドラインの更新と公開の維持とそのあり方について検討することを目的とした．

B．研究方法

2005年版と2009年版の肝癌診療ガイドライン発表後の公開手順と維持，公開状況について比較検討し，問題点について考察した．これら２つのガイドライン評価について外部評価AGREEによる評価を用い比較検討し，ガイドライン使用者に対するアンケート結果についても検討した．

（倫理面への配慮）

ガイドライン作成や公開に関わる情報のみを取り扱い個人情報は取り扱わないので倫理面について配慮すべき事象はない．

C．研究結果

（１）肝癌診療ガイドライン発表後の公開手順と維持について

ガイドラインの公開は，日本肝臓学会の学会ホームページ，日本肝癌研究会ホームページ， Minds医療情報サービス，日本癌治療学会のホームページ，国立がん研究センターホームページの5箇所に公開されていた．公開形式はガイドライン担当学会である肝臓学会ホームページへのリンクを貼っているものが，日本肝癌研究会と国立がん研究センターの2箇所，Minds医療情報サービスと日本癌治療学会のホームページでは独自の方法で公開されていた．

初版と改訂版の公開時期の比較では，初版の冊子出版は2005年2月，その後2006年3月(13ヶ月後)にMindsのホームページと国立がん研究センターホームページに公開，さらに日本肝臓学会および日本研究会ホームページでの公開が2006年10月(20ヶ月)，ついでJournal of Gastroenterologyでの英語版の公開(治療アルゴリズムのみ)が2009年1月(47ヶ月)，日本癌治療学会ホームページでの公開が2009年3月(49ヶ月)，冊子出版からの公開までの平均期間は27ヶ月であった．

改訂第2版では，冊子出版が2009年11月，その後Hepatology Researchに英語版の公開が2010年6月(7か月)，日本肝臓学会および日本肝癌研究会ホームページでの公開が2010年12月(13ヶ月)，Mindsホームページと国立がん研究センターホームページでの公開が2011年3月(16ヶ月)，日本癌治療学会ホームページでの公開が2011年7月(20ヶ月)であった．冊子出版から公開までの平均期間は平均14ヶ月と短縮がみられた．

（２）ガイドライン評価について

改訂版のAGREEによる評価では6領域の評価項目毎の平均点および標準化スコアは，「対象と目的」3.78点，93%，「利害関係者の参加」2.79点，60%，「作製の厳格さ」3.71点，90%，「明確さと提示の方法」3.33点，78%，「適応可能性」2.56点，52%，「編集の独立性」3.08点，69%であった．2005年版の評価に比して，「明確さと提示の方法」について評価は大きくは変わっていないものの，それ以外の領域についてはいずれも2005年度版の評価を上回っていた．「対象と目的」，「作製の厳格さ」および「明確さと提示の方法」については優れていたが，「利害関係者の参加」及び「適応可能性」については改善の必要性が考えられた．これらの結果から改訂版では初版の外部評価の結果がある程度反映されていた．

改訂版にて行われたガイドライン利用者(医師)に対するアンケート結果では，肝癌診療で分からない時困った時のガイドライン2009年版利用率は69%と最も多く，

別添３

厚生労働科学研究費補助金
(分担研究報告書)

日常利用しているガイドラインでは95%がガイドライン2009年度版を利用していた．肝癌診療ガイドラインの利用媒体は80%近くが紙媒体，学会HPが40%，Mindsが約10%であった．利用頻度は月1－3回が44%と最も多く，次いで週1回以上が40%であった．最もよく利用するのは治療のアルゴリズムで96%，次いでサーベイランスのアルゴリズムであった．
治療方針への影響として14%に治療方針に変化があり，49%で治療方針に自信が持てたと回答があった．適切な改訂の頻度は，1年/1回が9%，2年/1回が35%，3年/1回が19%，4年/1回(現在のまま)が34%であった． 77%が重要なエビデンスが出現した場合に部分改訂を行うべきとう意見であった．

D．考察

肝癌診療ガイドラインの公開は，日本肝臓学会，日本肝癌研究会，Minds医療情報サービス，日本癌治療学会，国立がん研究センターの5箇所となっている．これは日本には，NCCNのようなガイドライン公開について中心となる機関が存在しないためであると考えられる．それぞれが独自の形式に変換して公開するために初版にて情報公開に遅延が生じていたが，改訂版では公開までの期間がと約半分に短縮していた．これは初版の公開時に時間のかかった箇所で，公開までの過程が確立されたためであった．しかしながら公開については，公開方法をネットワークのみにし，米国のNCCNのような中心となる機関の整備することにより迅速な情報の提供ができる可能性がある．

ガイドライン評価については，外部評価は初版の評価に比較しより良好な評価が得られており，初版の評価を反映し作製されたものと考えられた．評価が改善していない「利害関係者の参加」及び「適応可能性」に関しては，肝癌診療ガイドラインがエビデンスとその収集方法を厳格に守って作製されているというガイドラインの特徴の一つとも考えられた．

ガイドライン利用者に関するアンケートでは，ほとんどは紙媒体(出版物)による利用であり，最も利用されているものはアルゴリズムであった．ガイドラインによる医師の裁量制限や医療訴訟の増加を危惧する意見もあった．ガイドラインの各項目により分量やわかりやすさ，適切さの評価については，妥当であると考えられた．改訂については，エビデンスに応じての部分改訂を考える必要がある．

E．結論

肝癌診療ガイドライン公開状況の検討から，公開方法や中心となる機関(機構)の設置などの整備がさらに必要であると考えられる．ガイドラインの更新については，肝癌診療ガイドラインはエビデンスを重視するという特徴があるものの，AGREEのような客観性のある外部評価とともにアンケート調査のような利用者の意見を反映しつつ，更新していく必要があると考えられた．

F．健康危険情報

特記すべきことなし．

G．研究発表

1． 論文発表

(1) Mizukoshi E, Nakamoto Y, Arai K, Yamashita T, Sakai A, Sakai Y, Kagaya T, Yamashita T, Honda M, Kaneko S; Comparative analysis of various tumor-associated antigen-specific t-cell responses in patients with hepatocellular carcinoma. Hepatology 53: 1206-16, 2011.
(2) Nakamoto Y, Mizukoshi E, Kitahara M, Arihara F, Sakai Y, Kakinoki K, Fujita Y, Marukawa Y, Arai K, Yamashita T, Mukaida N, Matsushima K, Matsui O, Kaneko S; Prolonged recurrence-free survival following OK432-stimulated dendritic cell transfer into hepatocellul ar carcinoma during transarterial embolization. Clin Exp Immunol 163: 165-77, 2011.
(3) Sunagozaka H, Honda M, Yamashita T, Nishino R, Takatori H, Arai K, Yamashita T, Sakai Y, Kaneko S; Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma. Int J Cancer 129: 1576-85, 2011.
(4) Yamashita T, Honda M, Kaneko S; Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection. J Gastroenterol Hepatol 26: 960-4, 2011.
(5) Takata Y, Nakamoto Y, Nakada A, Terashima T, Arihara F, Kitahara M, Kakinoki K, Arai K, Yamashita T, Sakai Y, Yamashita T, Mizukoshi E, Kaneko S; Frequency of CD45RO+ subset in CD4+CD25(high) regulatory T cells associated with progression of hepatocellular carcinoma. Cancer Lett 307: 165-73, 2011.

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（分担研究報告書）

(6) Takatori H, Yamashita T, Honda M, Nishino R, Arai K, Yamashita T, Takamura H, Ohta T, Zen Y, Kaneko S: dUTP pyrophosphatase expression correlates with a poor prognosis in hepatocellular carcinoma. Liver Int 30: 438-446, 2010
(7) Yamashita T, Honda M, Nio K, Nakamoto Y, Yamashita T, Takamura H, Tani T, Zen Y, Kaneko S: Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation. Cancer Res 70: 4687-4697, 2010
(8) Iida N, Nakamoto Y, Baba T, Nakagawa H, Mizukoshi E, Naito M, Mukaida N, Kaneko S: Antitumor effect after radiofrequency ablation of murine hepatoma is augmented by an active variant of CC Chemokine ligand 3/macrophage inflammatory protein-1alpha. Cancer Res 70: 6556-6565, 2010
(9) Hodo Y, Hashimoto S, Honda M, Yamashita T, Suzuki Y, Sugano S, Kaneko S, Matsushima K. Comprehensive gene expression analysis of 5'-end of mRNA identified novel intronic transcripts associated with hepatocellular carcinoma. Genomics 95: 217-223, 2010
(10)Yamashita T, Honda M, Takatori H, Nishino R, Minato H, Takamura H, Ohta T, Kaneko S: Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma. J Hepatol 50: 100-110, 2009
(11)Mizukoshi E, Nakamoto Y, Arai K, Yamashita T, Mukaida N, Matsushima K, Matsui O, Kaneko S: Enhancement of tumor-specific T cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma. Int J Cancer, in press, 2009
(12)科学的根拠に基づく肝癌診療ガイドライン　2009年版，56－61，96－109，2009
(13)山下竜也，金子周一：肝腫瘍．内科学書　改訂第7版：280－286，2008

2.　学会発表
１．山下竜也，寺島健志，山下太郎，菅金子周一，進行肝細胞癌に対するIFN併用5-FU肝動注化学療法におけるサイトカインプロファイルによる奏効および予後因子の検討，第97回日本消化器病学会総会，パネルディスカッション，２０１１
２．山下竜也，寺島健志，金子周一，当科における進行肝細胞癌に対するソラフェニブ療法の検討，第47回日本肝癌研究会，シンポジウム，２０１１
３．山下竜也，荒井邦明，金子周一，当科における肝癌初発治療法と肝癌診療ガイドライン治療アルゴリズムの比較，第39回日本肝臓学会西部会，シンポジウム，２０１１
４．Shuichi Kaneko1, Junji Furuse2, Kenji Ikeda3, Masatoshi Kudo4, The Study Group on New Liver Cancer Therapies (NLCT), Efficacy and safety of sorafenib for hepatocellular carcinoma、: a multi-center retrospective study in Japan, The 62nd Annual Meeting of the American Association for the Study of Liver Disease (AASLD), 2011
５．山下竜也，寺島健志，金子周一，当科における進行肝細胞癌に対するIFN併用5-FU肝動注化学療法を中心とした集学的治療の現況，第96回日本消化器病学会総会，パネルディスカッション，２０１０
６．山下竜也，寺島健志，金子周一，当科における進行肝細胞癌予後改善を目指した肝動注化学療法を中心とした集学的治療，第46回日本肝癌研究会，シンポジウム，２０１０
６．山下竜也，荒井邦明，金子周一，当科における肝癌初発治療法と各種治療アルゴリズムの比較，第95回日本消化器病学会総会，シンポジウム６，２００９
７．山下竜也，金子周一，肝癌診療ガイドライン改訂　化学療法，第45回日本肝臓学会総会，特別企画「肝癌診療ガイドラインの改訂公開シンポジウム」，２００９
８．荒井邦明，金子周一，工藤正俊，肝癌診療ガイドライン改訂　診断(ＵＳ)，第45回日本肝臓学会総会，特別企画「肝癌診療ガイドラインの改訂公開シンポジウム」，２００９

H．知的財産権の出願・登録状況
１．特許取得
該当事項なし

２．実用新案登録
該当事項なし

３．その他

Comparative Analysis of Various Tumor-Associated Antigen-Specific T-Cell Responses in Patients with Hepatocellular Carcinoma

Eishiro Mizukoshi, Yasunari Nakamoto, Kuniaki Arai, Tatsuya Yamashita, Akito Sakai, Yoshio Sakai, Takashi Kagaya, Taro Yamashita, Masao Honda, and Shuichi Kaneko

Many tumor-associated antigens (TAAs) recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials. However, there are very few in the field of immunotherapy for hepatocellular carcinoma (HCC) because there have not been comparative data regarding CTL responses to various TAAs. In the present study, using 27 peptides derived from 14 different TAAs, we performed comparative analysis of various TAA-specific T-cell responses in 31 HCC patients to select useful antigens for immunotherapy and examined the factors that affect the immune responses to determine a strategy for more effective therapy. Twenty-four of 31 (77.4%) HCC patients showed positive responses to at least one TAA-derived peptide in enzyme-linked immunospot assay. The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, multidrug resistance-associated protein (MRP) 3, alpha-fetoprotein (AFP) and human telomerase reverse transcriptase (hTERT) were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced *de novo* T-cell responses to lymphocyte-specific protein tyrosine kinase, human epidermal growth factor receptor type 2, p53, and hTERT. Blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) resulted in unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles of peripheral blood mononuclear cells stimulated by TAA-derived peptides. *Conclusion:* Cyclophilin B, SART2, SART3, p53, MRP3, AFP, and hTERT were immunogenic targets for HCC immunotherapy. TAA-specific immunotherapy combined with HCC treatments and anti-CTLA-4 antibody has the possibility to produce stronger tumor-specific immune responses. (HEPATOLOGY 2011;53:1206-1216)

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and becoming an important public health concern.[1,2] Although many kinds of treatments have been performed for HCC, their effects are limited because the recurrence rate of HCC is very high; therefore, the development of new therapeutic options to prevent recurrence is necessary.[3,4]

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Many tumor-associated antigens (TAAs) and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials for several cancers.[5-21] The epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials.[22,23] With regard to immunotherapy for HCC, few kinds of TAAs and their epitopes have been used and only clinical data of α-fetoprotein (AFP) have been reported.[24,25] In human trials targeting AFP, it is possible to raise an AFP-specific T-cell response using AFP-derived peptides, but this has shown little

Abbreviations: AFP, alpha-fetoprotein; CTL, cytotoxic T cell; ELISPOT, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFN, interferon; Lck, lymphocyte-specific protein tyrosine kinase; MRP, multidrug resistance-associated protein; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen.

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antitumor effect. On the other hand, immunotherapy trials using autologous tumor lysate or dendritic cells have shown statistically significant improvements in the risk of HCC recurrence and recurrence-free survival.[26] These reports suggest that tumor antigen-specific immunotherapy is effective to reduce the recurrence rate after HCC treatment; therefore, it is necessary to find immunogenic antigens or their epitopes to develop more effective immunotherapy.

In addition, in the field of molecular targeting therapies, developments of monoclonal antibodies targeting immunomodulatory molecules to enhance antitumor immunity are progressing and some of these are under clinical trial.[27] In particular, clinical data of anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody have shown durable objective response and stable disease in melanoma patients.[28]

In the present study we performed comparative analysis of various TAA-specific T-cell responses in patients with HCC and examined the factors that affect the immune responses, including anti-CTLA-4 antibody. This approach offers useful information to select immunogenic TAAs and to develop a new strategy for HCC immunotherapy.

Patients and Methods

Patients and Laboratory Testing. In this study we examined 31 human leukocyte antigen (HLA)-A24-positive patients with HCC, 29 chronic hepatitis C patients without HCC, who were diagnosed by liver biopsy, and 11 healthy blood donors who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody (Ab). The diagnosis of HCC was histologically confirmed in 21 patients. For the remaining 10 patients the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[29]

HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described.[19] The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[30] The severity of liver disease was evaluated according to the criteria of Desmet et al.[31] using biopsy specimens of liver tissue.

All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

Table 1. Peptides

Peptide No.	Peptide Name	Source	Reference	Amino Acid Sequence	Number of Specific Spots in Normal Donors (Mean SD)
1	$ART1_{188}$	ART1	5	EYCLKFTKL	0.9 ± 1.1
2	$ART4_{161}$	ART4	6	AFLRHAAL	0.3 ± 0.5
3	$ART4_{899}$	ART4	6	DYPSLSATDI	0.6 ± 1.0
4	$Cyp\text{-}B_{109}$	Cyp-B	7	KFHRVIKDF	0.5 ± 0.9
5	$Cyp\text{-}B_{315}$	Cyp-B	7	DFMIQGGDF	1.2 ± 1.7
6	Lck_{208}	Lck	8	HYTNASDGL	0.3 ± 0.6
7	Lck_{486}	Lck	8	TFDYLRSVL	0.2 ± 0.8
8	Lck_{488}	Lck	8	DYLRSVLEDF	0.9 ± 1.5
9	$MAGE1_{135}$	MAGE-A1	9	NYKHCFPEI	1.0 ± 0.9
10	$MAGE3_{195}$	MAGE-A3	10	IMPKAGLLI	1.4 ± 1.7
11	$SART1_{1690}$	SART1	11	EYRGFTQDF	0.9 ± 1.3
12	$SART2_{899}$	SART2	12	SYTRLFLIL	1.0 ± 1.4
13	$SART3_{109}$	SART3	13	VYDYNCHVDL	2.1 ± 1.9
14	$Her\text{-}2/neu_8$	Her-2/neu	14	RWGLLLALL	1.4 ± 2.0
15	$p53_{125}$	p53	15	TYSPALNKMF	1.4 ± 1.5
16	$p53_{161}$	p53	16	AIYKQSQHM	0.4 ± 0.6
17	$p53_{204}$	p53	17	EYLDDRNTF	1.1 ± 1.5
18	$p53_{211}$	p53	17	TFRHSVVV	0.9 ± 1.9
19	$p53_{235}$	p53	17	NYMCNSSCM	2.1 ± 2.6
20	$MRP3_{503}$	MRP3	18	LYAWEPSFL	0.2 ± 0.5
21	$MRP3_{692}$	MRP3	18	AYVPQQAWI	1.5 ± 2.1
22	$MRP3_{765}$	MRP3	18	VYSDADIFL	0.9 ± 1.0
23	AFP_{357}	AFP	19	EYSRRHPQL	1.8 ± 2.0
24	AFP_{403}	AFP	19	KYIQESQAL	1.1 ± 1.5
25	AFP_{434}	AFP	19	AYTKKAPQL	0.8 ± 1.1
26	$hTERT_{167}$	hTERT	20	AYQVCGPPL	0.8 ± 1.1
27	$hTERT_{324}$	hTERT	20	VYAETKHFL	0.5 ± 0.7
28	HIV env_{584}	HIV env	32	RYLRDQQLL	1.3 ± 2.0
29	HCV $NS3_{1031}$	HCV NS3	33	AYSQQTRGL	ND
30	CMV $pp65_{328}$	CMV pp65	34	QYDPVAALF	13.3 ± 15.7

ND, not determined.

Peptides, Cell Lines, and Preparation of PBMCs. Twenty-seven peptides derived from 14 different TAAs (Table 1), human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$),[32] HCV NS3-derived peptide ($HCVNS3_{1031}$),[33] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$),[34] which were identified as HLA-A24 restricted CTL epitopes in previous studies, were used. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry and their purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS.[35] PBMCs were isolated before HCC treatments as described.[20] In 12 patients their PBMCs were also obtained 4 weeks after treatments.

Table 2. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Child Pugh (A/B/C)	Diff. Degree* (wel/mod/ por/ND)	Tumor Size† (large/small)	Tumor Multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/ IIIC/IV)
Normaldonors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
Chronichepatitis	29	16/13	59 ± 10	92 ± 94	31 ± 87	27/2/0	ND	ND	ND	ND	ND
HCC	31	23/8	71 ± 4	74 ± 33	1768 ± 9103	20/10/1	11/10/0/10	22/9	20/11	9/22	10/12/3/1/2/3

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
†Tumor size was divided into either "small" (≦2 cm) or "large" (>2 cm).

CTL Induction and Cytotoxicity Assay. CTL induction and cytotoxicity assays were performed as described.[20] Briefly, stimulated PBMCs were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1, and 3:1. In cases where the number of CTLs was insufficient, cytotoxicity assays were performed at effector to target ratios less than 100:1.

Interferon Gamma IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. IFN-γ ELISPOT assays were performed as reported.[20] Responses to TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviations (SDs) of the baseline response detected in 11 normal blood donors (Table 1), and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Responses to HIV-, HCV-, and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. In ELISPOT assay with blocking CTLA-4, anti-human CTLA-4 (eBioscience, Tokyo, Japan) was added at a final concentration of 50 μg/mL, which has been described to have maximum effect in *in vitro* cultures.[36] As a control, functional grade mouse immunoglobulin G (IgG)2a isotype control was used. The assay with blocking CTLA-4 was performed in triplicate and the results were statistically analyzed using the unpaired Student's *t* test.

Cytokine and Chemokine Profiling. The effect of CTLA-4 antibody on TAA-specific T-cell responses was also analyzed by cytokine and chemokine profiling. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay (Bio-Rad, Hercules, CA). These included interleukin (IL)-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were carried out using Bio-plex Manager software v. 4.1.1.

Cytokine Secretion Assay. TAA-specific IFN-γ-producing T cells were also analyzed by cytokine secretion assay. The assay was performed with the MACS cytokine secretion assay (Miltenyi Biotec K.K., Tokyo, Japan), in accordance with the manufacturer's instructions. Briefly, 5,000,000 PBMCs were pulsed with TAA-derived peptides for 16 hours and then incubated with 20 μL of IFN-γ detection antibody, 10 μL of anti-CD8-APC Ab (Becton Dickinson, Tokyo, Japan), 10 μL of anti-CCR7-FITC Ab (eBioscience, Tokyo, Japan), and 10 μL of anti-CD45RA-PerCP-Cy5.5 Ab (eBioscience, Tokyo, Japan) for 10 minutes at 4°C. After washing with a cold buffer (phosphate-buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were resuspended with 500 μL of cold buffer and analyzed using FACSCalibur (Becton Dickinson, Tokyo, Japan). As a positive control, $CMVpp65_{328}$-specific IFN-γ-producing T cells were also analyzed by the same methods. The number of IFN-γ-producing T cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs.

Results

Patient Profile. The clinical profiles of the 11 healthy blood donors, 29 patients with chronic hepatitis C, and 31 patients with HCV-related HCC analyzed in the present study are shown in Table 2 and Fig. 1. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th v.), 10, 12, 3, 1, 2, and 3 patients were classified as having stage I, II, IIIA, IIIB, IIIC, and IV tumors, respectively.

Detection of TAA-Specific T Cells in HCC Patients. First we examined the frequency of cells that specifically reacted with TAA-derived and control peptides in HCC patients. Fifty-one responses in total were observed against TAA-derived peptides. Twenty-

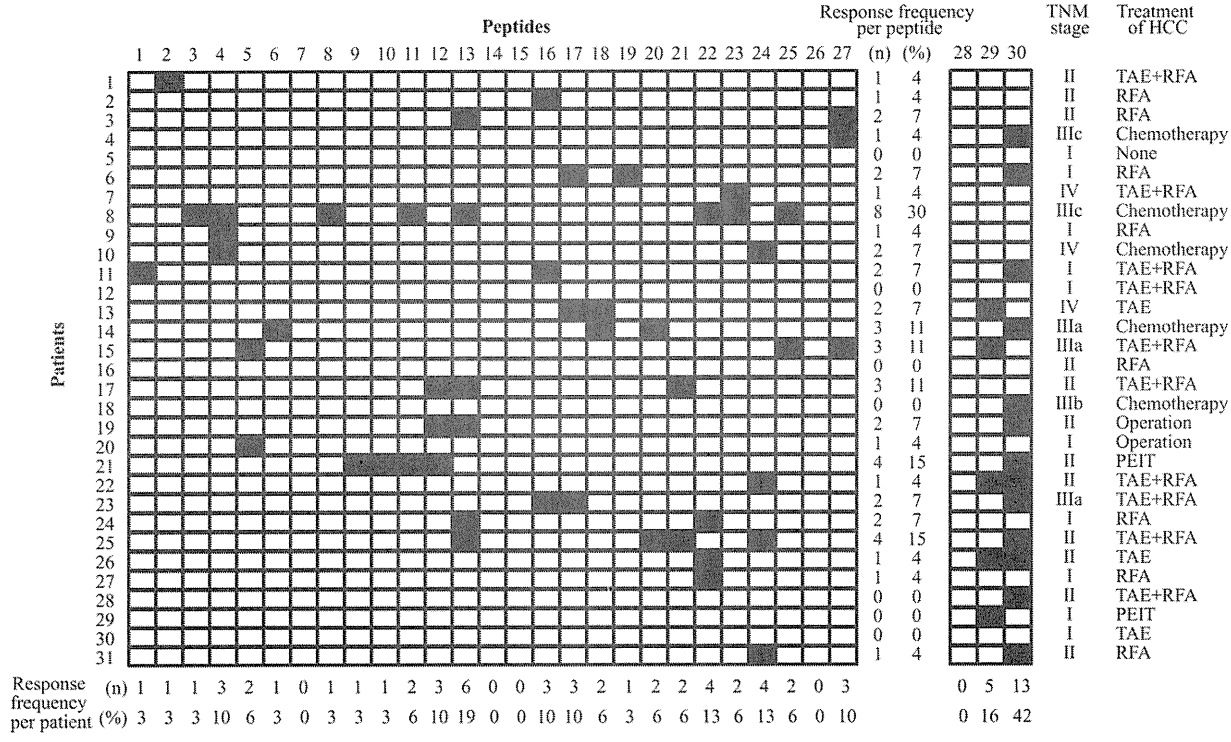

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate the presence of a significant IFN-γ T-cell response to peptides. Peptide sequences are described in Table 1 and characteristics of patients in Table 2.

four of 31 (77.4%) patients showed positive responses to at least one TAA-derived peptide and most of them showed responses to 1 to 4 kinds of TAA-derived peptide. Twenty-three of 27 (85.2%) TAA-derived peptides were recognized by T cells of at least one patient. Peptides 4, 12, 13, 16, 17, 22, 24, and 27 were recognized in more than two patients, suggesting that these peptides were immunogenic. Peptides 28 (HIV env_{584}), 29 (HCV $_{1031}$), and 30 (CMV $pp65_{328}$) were recognized by 0 (0%), 5 (16%), and 13 (42%) patients, respectively.

The magnitude of TAA-specific T-cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population (Fig. 2A). The range of TAA-derived peptide-specific T-cell frequency was 10-60.5 cells/300,000 PBMCs. Those specific to peptides 13 and 16 numbered more than 30 cells/300,000 PBMCs, suggesting that these peptides were immunogenic. The frequencies of T cells specific to HCV- and CMV-derived peptides were 12-22 cells and 12-92/300,000 PBMCs, respectively.

Whether these TAA-derived peptides were capable of generating peptide-specific CTLs from PBMCs was investigated in HCC patients. The seven peptides were selected according to the magnitude of TAA-specific T-cell responses determined by the frequency of T cells with a positive response. The CTLs generated with these peptides were cytotoxic to C1RA24 cells pulsed with the corresponding peptides (Fig. 2B).

Comparison of TAA-Specific T-Cell Responses Between the Patient Groups With and Without HCC. To characterize the immunogenicity and specificity of TAA-derived peptides, we compared T-cell responses to the peptides derived from TAA, HIV, HCV, and CMV among three groups consisting of normal blood donors, patients with chronic hepatitis C, and patients with HCV-related HCC. A significant TAA-specific T-cell response was not detected in normal blood donors (Fig. 3A). A response was detected in both chronic hepatitis C and HCC patient groups, but it was more frequently observed in HCC patients. HIV-specific T-cell response was not detected in any group. HCV-specific T-cell response rate was not different between the groups with chronic hepatitis C and HCC. CMV-specific T-cell response rates were similar among the three groups. Similar tendencies were observed in the analysis of individual peptides (Fig. 3B). We also examined the frequency of T cells responsive to peptides among the three groups. The mean frequency of TAA-specific T cells without *in vitro* expansion was higher in HCC patients than in

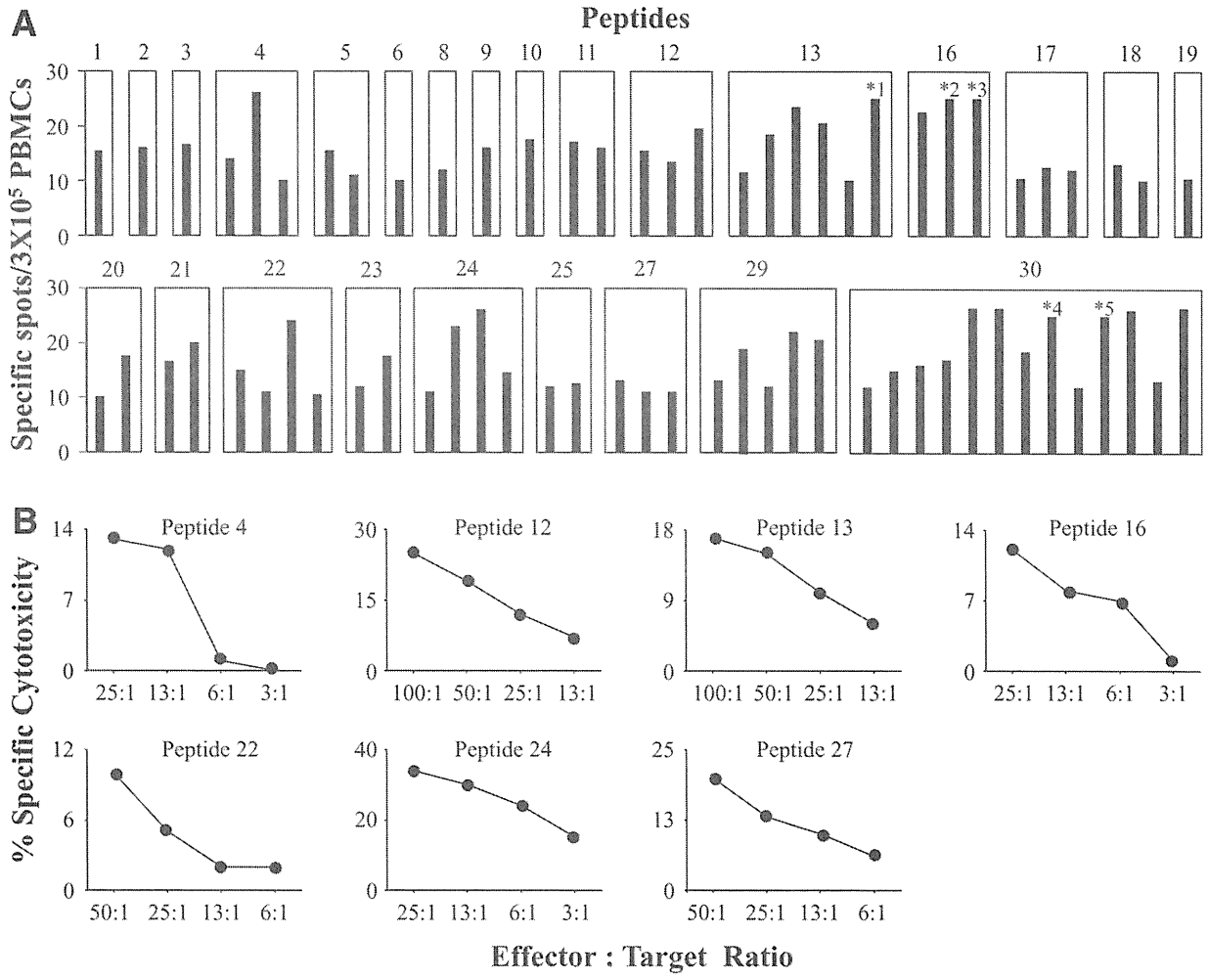

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, and 67.5 specific spots, respectively. (B) Representative TAA-specific T-cell responses were also analyzed by CTL assay. T cell lines were generated from PBMC of the HLA-A24-positive HCC patients by stimulation with TAA-derived peptides (peptides 4, 12, 13, 16, 22, 24, and 27) (see Table 1). Expanded T cell lines were then tested for specific cytotoxicity against the corresponding peptides in a standard ^{51}Cr release assay at the indicated E:T ratios.

patients with chronic hepatitis C for 14 of 27 TAA-derived peptides (peptides 1, 2, 3, 4, 12, 16, 18, 19, 20, 21, 22, 24, 25 and 27) (Fig. 3C).

Enhancement of TAA-Specific T-Cell Responses After HCC Treatments. Several studies including our own have clarified that HCC treatments enhanced HCC-specific immune responses (19, 37, 38). In this study, we examined whether the enhancement was observed equally in all kinds of TAAs or specifically in some TAAs. For this purpose we measured the frequency of TAA-specific T cells before and after HCC treatment by ELISPOT assay in 12 cases who received transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), or chemotherapy. The frequency of TAA-specific T cells increased in all patients and it was observed for 23 of 27 TAA-derived peptides (Fig. 4A). The enhancement was observed in the patients who received TAE, RFA, or chemotherapy and even in the patients without an increase in the frequency of CMV-specific T cells. Peptides 7, 14, 15, and 26, which were not recognized by T cells in all HCC patients before treatments (Fig. 1), were recognized by T cells in 1, 4, 1, and 5, respectively, of 12 patients after treatments. Representative results of enhancement of TAA-specific immune responses are shown in Fig. 4B. The frequency of TAA-specific T cells increased to 11-80 cells/300,000 PBMCs after treatments.

The enhancement of TAA-specific immune responses was also confirmed by cytokine secretion assay. Representative results are shown in Fig. 4C. In this patient (patient 25) the frequency of TAA-specific IFN-γ-producing $CD8^+$ T cells was increased from 0.4% to 1.4% of $CD8^+$ T cells after HCC treatment.

A

	TAA	HIV antigen	HCV antigen	CMV antigen
Normal donors	0/11(0%)	0/11(0%)	ND	4/11(36%)
Patients with HCV	10/29(34%)	0/29(0%)	8/29(28%)	15/29(52%)
Patients with HCC	24/31(77%)	0/31(0%)	5/31(16%)	13/31(42%)

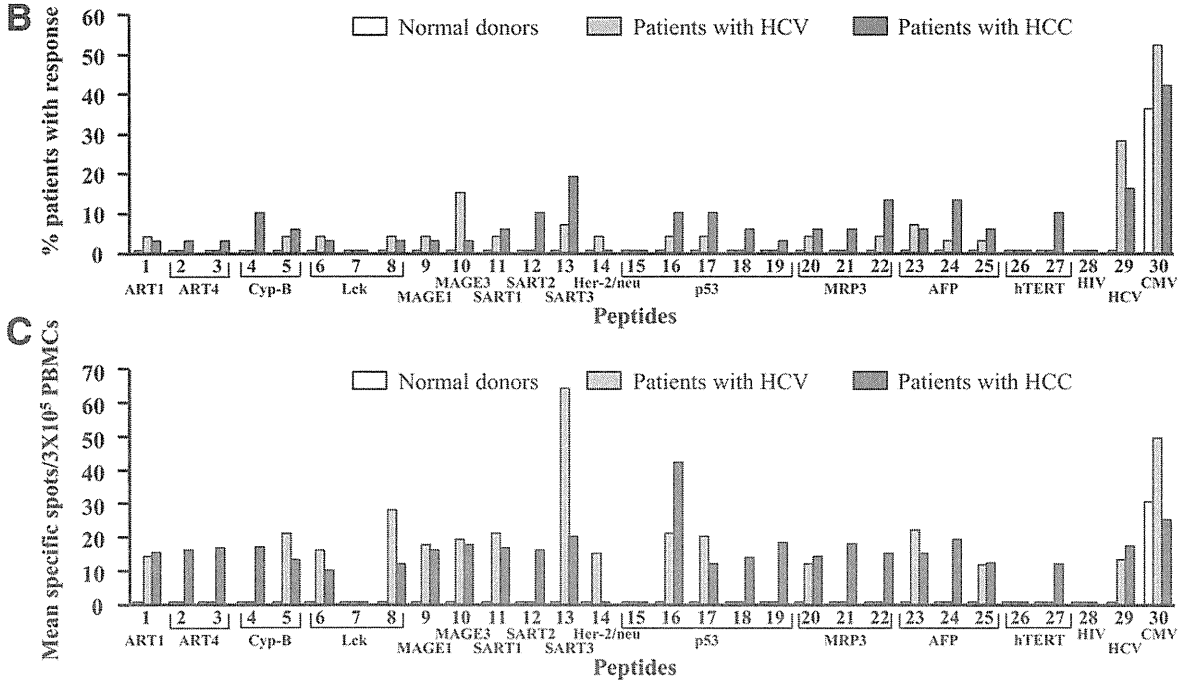

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response to tumor-associated, HIV, HCV, and CMV antigens in each group. (B) Graph shows the percentage of patients in each group who showed a significant IFN-γ T-cell response to individual peptides. Peptide sequences are described in Table 1. (C) Mean frequency of peptide-specific IFN-γ-producing T cells in each group. The frequency of IFN-γ-producing T cells was analyzed by ELISPOT assay.

In this assay we also examined the naïve/effector/memory phenotype of these cells by the criterion of CD45RA/CCR7 expression.[39] Phenotypic analysis of TAA-specific, IFN-γ-producing memory $CD8^+$ T cells before and after treatment showed that the frequency of $CD45RA^-/CCR7^+$ central memory T cells was the highest, indicating that the posttherapeutic increase in these T cells is due to the increase in cells with this phenotype (Fig. 4D). In this patient the number of T cells with the $CD45RA^-/CCR7^+$ phenotype increased from 73 cells/300,000 PBMCs before treatment to 316 cells/300,000 PBMCs after treatment. Similar results were noted in five patients.

Blocking CTLA-4 Restores TAA-Specific T-Cell Responses. In previous studies including our own,[19,20,24] the CTL epitopes that correlate with the prevention of tumor progression or prognosis of HCC patients have not been identified. One of the reasons for this is considered to be that the naturally occurring T-cell responses to the epitopes are weak; therefore, recent tumor immunotherapeutic studies are moving toward modulation of T-cell responses.

CTLA-4 is recognized as a critical negative regulator of immune response; therefore, its blockade has been considered to contribute to antitumor activity.[27] In a recent study it was reported that blocking of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of CTLA-4 antibodies.[40] To examine whether similar occurs for immune response in HCC patients, we analyzed 32 separate TAA-specific T-cell responses in 15 HCC patients using 13 TAA-derived peptides. Incubation of T cells with CTLA-4 antibodies resulted in an increase of the number of TAA-specific T cells in 18 of 32 (56%) responses and in 9 of 15 (60%) patients (Fig. 5A). Fourteen and four patients showed increases of 1-10 and more than 10 TAA-specific T cells, respectively. Representative results of six patients are shown

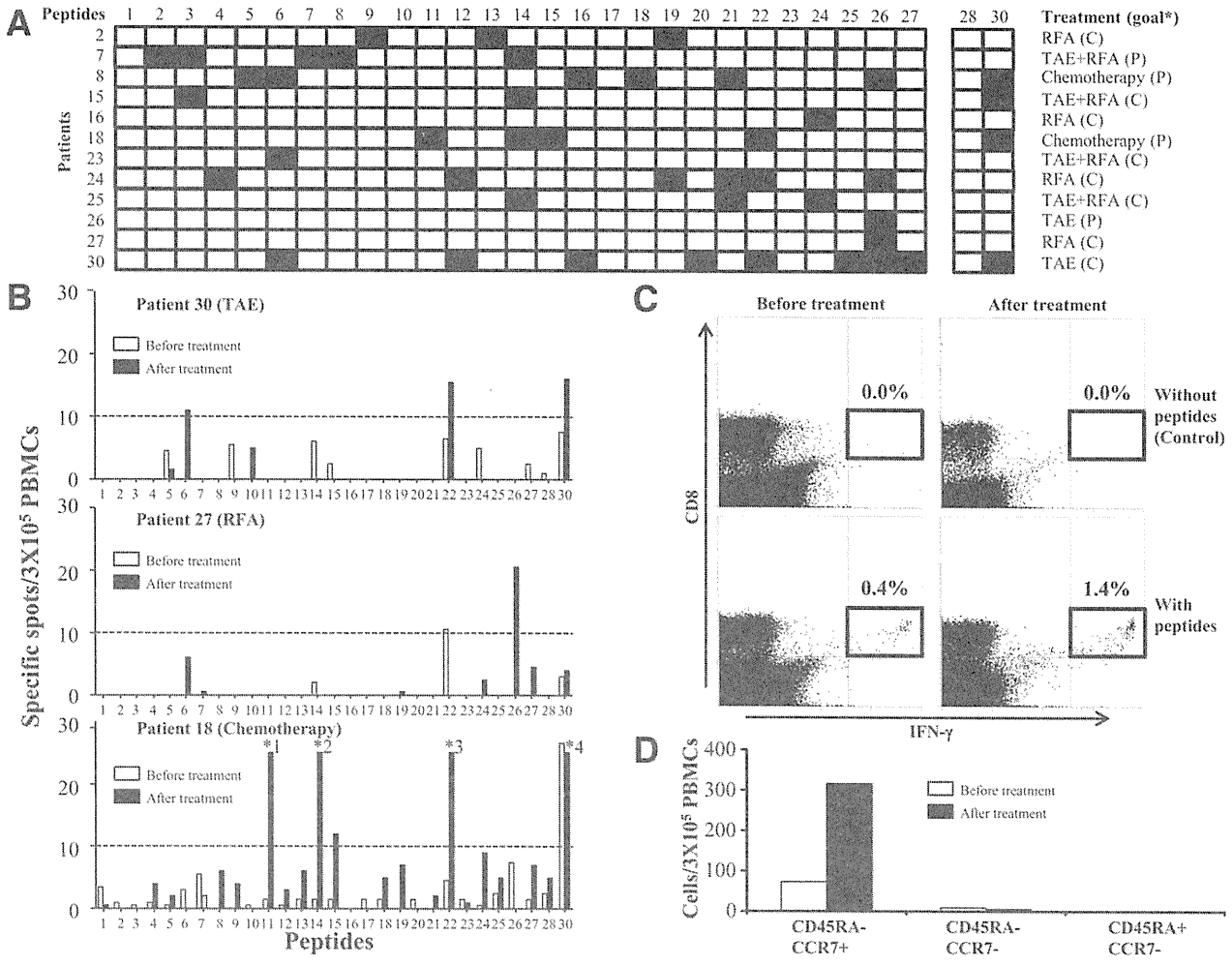

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-

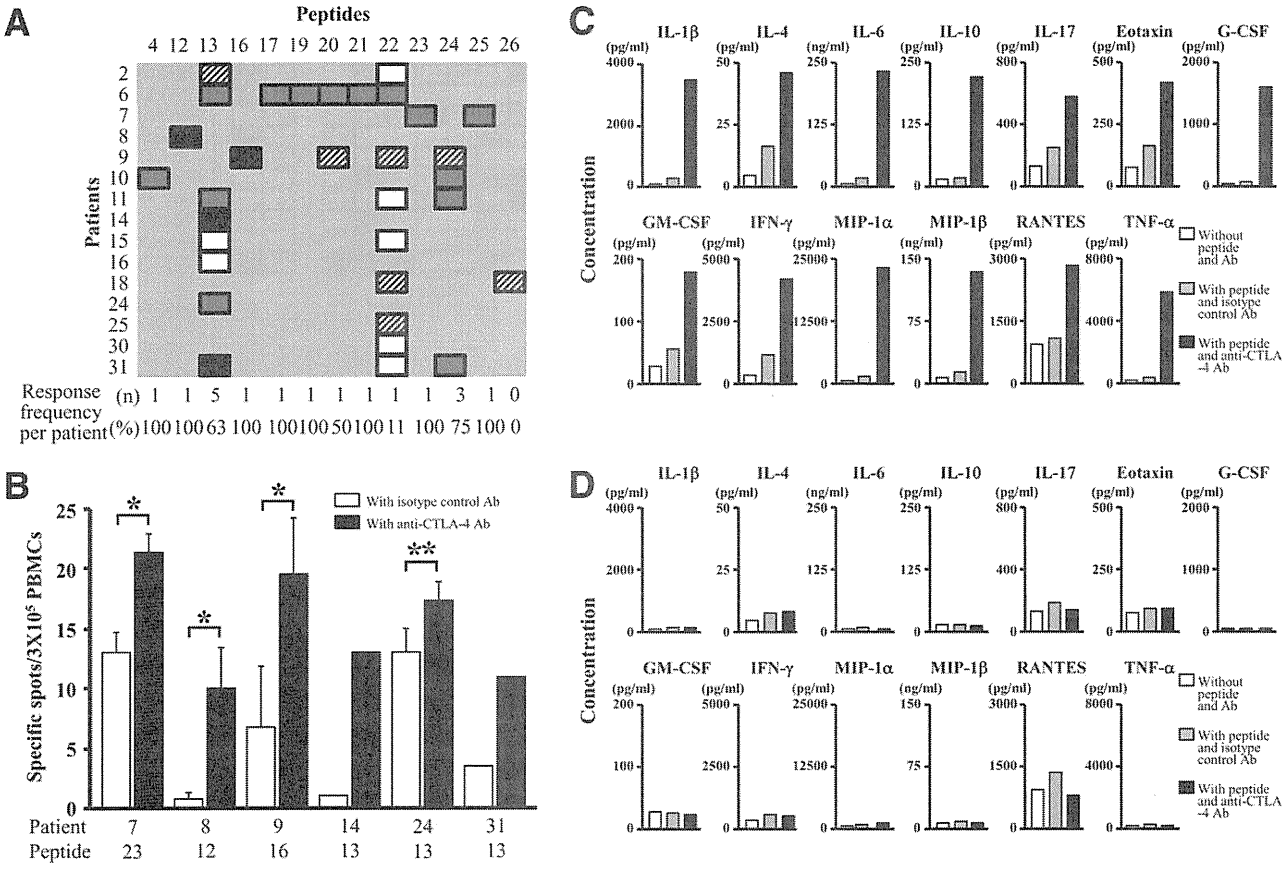

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^{-}/CCR7^{+}$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.

On the other hand, recent studies have shown the important role of $CD4^+$ helper T cells in optimal function and proliferation of $CD8^+$ T cells.[46] Therefore, the lack of $CD4^+$ helper T cells or anergic $CD4^+$ T cells may explain the limited TAA-specific $CD8^+$ T-cell responses in HCC. Further studies using $CD4^+$ T-cell-depleted PBMCs or $CD8^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

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References

1. Deuffic S, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. Lancet 1998;351:214-215.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153-156.
3. Lencioni R. Loco-regional treatment of hepatocellular carcinoma. HEPATOLOGY 2010;52:762-773.
4. Okuwaki Y, Nakazawa T, Shibuya A, Ono K, Hidaka H, Watanabe M, et al. Intrahepatic distant recurrence after radiofrequency ablation for a single small hepatocellular carcinoma: risk factors and patterns. J Gastroenterol 2008;43:71-78.
5. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S. A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma. Cancer Res 2000;60:4830-4837.
6. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, et al. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor-infiltrating lymphocytes of lung cancer. Cancer Res 2000;60:3550-3558.
7. Gomi S, Nakao M, Niiya F, Imamura Y, Kawano K, Nishizaka S, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs. J Immunol 1999;163:4994-5004.
8. Harashima N, Tanaka K, Sasatomi T, Shimizu K, Miyagi Y, Yamada A, et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur J Immunol 2001;31:323-332.
9. Fujie T, Tahara K, Tanaka F, Mori M, Takesako K, Akiyoshi T. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. Int J Cancer 1999;80:169-172.
10. Nishiyama T, Tachibana M, Horiguchi Y, Nakamura K, Ikeda Y, Takesako K, et al. Immunotherapy of bladder cancer using autologous dendritic cells pulsed with human lymphocyte antigen-A24-specific MAGE-3 peptide. Clin Cancer Res 2001;7:23-31.
11. Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H, et al. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer 1999;81:459-466.
12. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J Immunol 2000;164:2565-2574.
13. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59:4056-4063.
14. Tanaka H, Tsunoda T, Nukaya I, Sette A, Matsuda K, Umano Y, et al. Mapping the HLA-A24-restricted T-cell epitope peptide from a tumour-associated antigen HER2/neu: possible immunotherapy for colorectal carcinomas. Br J Cancer 2001;84:94-99.
15. Eura M, Chikamatsu K, Katsura F, Obata A, Sobao Y, Takiguchi M, et al. A wild-type sequence p53 peptide presented by HLA-A24 induces cytotoxic T lymphocytes that recognize squamous cell carcinomas of the head and neck. Clin Cancer Res 2000;6:979-986.
16. Umano Y, Tsunoda T, Tanaka H, Matsuda K, Yamaue H, Tanimura H. Generation of cytotoxic T-cell responses to an HLA-A24 restricted epitope peptide derived from wild-type p53. Br J Cancer 2001;84:1052-1057.
17. Ferries E, Connan F, Pages F, Gaston J, Hagnere AM, Vieillefond A, et al. Identification of p53 peptides recognized by CD8(+) T lymphocytes from patients with bladder cancer. Hum Immunol 2001;62:791-798.
18. Yamada A, Kawano K, Koga M, Matsumoto T, Itoh K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes. Cancer Res 2001;61:6459-6466.
19. Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T, Kaneko S. Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma. Int J Cancer 2006;118:1194-1204.
20. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, et al. Cytotoxic T-cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. HEPATOLOGY 2006;43:1284-1294.
21. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415-2432.
22. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321-327.
23. Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. Cancer Sci 2006;97:970-976.
24. Butterfield LH, Ribas A, Meng WS, Dissette VB, Amarnani S, Vu HT, et al. T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 2003;9:5902-5908.
25. Butterfield LH, Ribas A, Dissette VB, Lee Y, Yang JQ, De la Rocha P, et al. A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 2006;12:2817-2825.
26. Butterfield LH. Recent advances in immunotherapy for hepatocellular cancer. Swiss Med Wkly 2007;137:83-90.
27. Dougan M, Dranoff G. Immune therapy for cancer. Annu Rev Immunol 2009;27:83-117.
28. O'Day SJ, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, Bondarenko IN, et al. Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Ann Oncol 2010;21:1712-1717.
29. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037-1043.