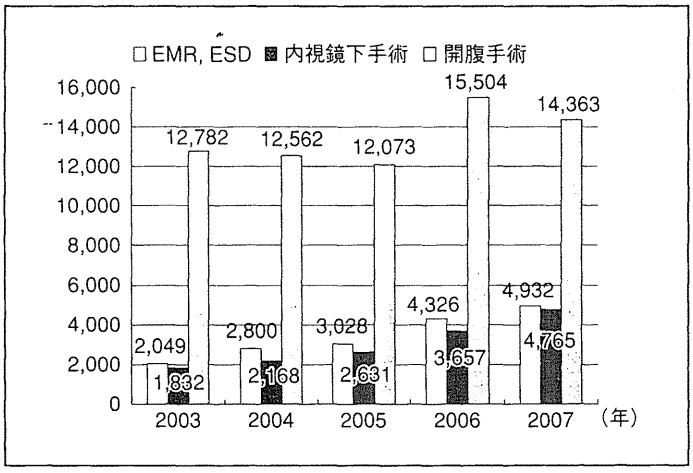

図1　胃癌治療法の変化

(文献1より引用)

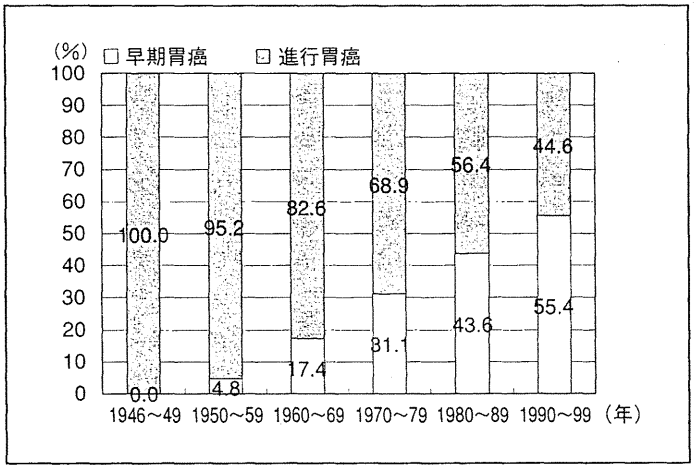

図2　増加する早期胃癌（癌研究会）

■ 高い胃癌診断技術レベル ■

2重造影に代表される胃癌のX線診断技術は，わが国の熱心な研究者により世界一のレベルに達した．また，内視鏡に先だってわが国で開発されたガストロカメラは，きわめて独創的な発明であった．もしファイバースコープの開発が遅れていれば，この技術が世界に広く普及した可能性がある．ファイバースコープを消化管内部を覗く機器としてではなく，ガストロカメラをより正確に活用するために用いたガストロファイバースコープには，わが国の診断グループの意地が感じられる．ファイバースコープが精細になり，ガストロカメラ（先端内蔵カメラ）はその存在意義が薄くなったが，その後開発されたビデオ内視鏡は，ビデオカメラを飲み込むという，いわばガストロカメラの発想に立ち戻ったものともいえよう．

また，東北大学の黒川利雄によって宮城県で開始されたX線による胃癌集団検診は，その後の早期胃癌の発見に大いに貢献した．その結果，わが国の胃癌症例における早期胃癌の割合は年々増加し，現在では全国的にほぼ50%を超えている．われわれの施設においても，1990年代には早期癌の占める割合は50%を超えている（図2）．このよ

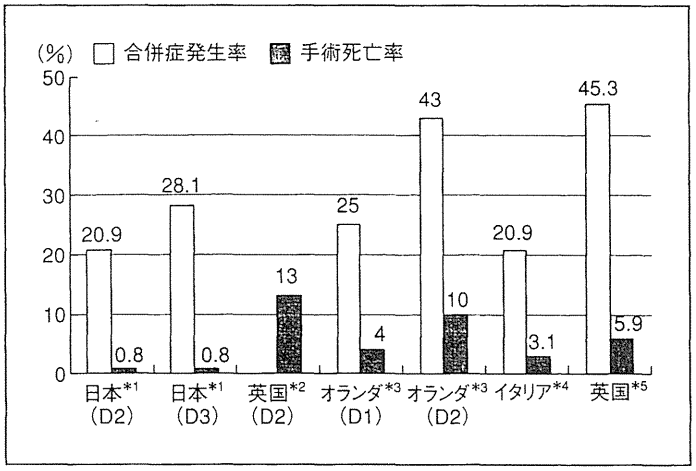

図3　胃癌手術の合併症発生率と手術死亡率の国際比較
＊1：JCOG9501[4]，＊2：Cuschieri[5]，＊3：Bonenkamp[6]，＊4：Degiuli[7]，＊5：MAGIC trial[8]

うに胃癌の早期発見の推進と，その結果得られた治療成績の向上に，わが国の診断グループの果たした役割はきわめて大きい．また，早期胃癌の増加は，それまで手術一辺倒であった胃癌の治療に，内視鏡治療や腹腔鏡下手術などの，QOL を重視した機能温存治療の発展をもたらした．これらの新しい治療においても，わが国は世界をリードする立場にあるといってよい．

■ 外科治療 ■

Billroth が 1881 年に胃癌の切除に成功したことは広く知られているが，それが胃癌の根治を目指したものではなく，胃癌による狭窄を解除する目的の姑息的な手術であったということは十分認識されていない．わが国では，梶谷鐶が癌の根治手術という概念を打ち立て，それを目指して努力が重ねられてきた．胃癌の手術後の局所再発は，胃癌と切除断端との距離を浸潤型では 5 cm 以上，限局型では 3 cm 以上取ることで減少した．術前の精緻な検査による胃癌の浸潤範囲の同定と深達度の推定は，わが国の手術の質を高めるのに大いに役立ったといえる．

胃癌研究会が結成され，胃癌取扱い規約が作られたことで，日本全国の研究施設や病院が共通の言語で治療成績を論じることができるようになった．年 2 回の研究会ではテーマが設定され，全国から膨大なデータが発表された．1970 年代の初めには，リンパ節郭清は D2 が標準であり，拡大根治手術といわれた D3 郭清（旧分類）が必ずしも治療成績の向上に寄与しないことが明らかになっていた．しかしその後，「リンパ節の郭清範囲は広いほど治療成績がよくなるはずである」という前提で，郭清範囲を大動脈周囲リンパ節まで拡大する D4 郭清（現在の D3）が提唱された．リンパ節郭清に熱心な施設では相当の症例数で D4 郭清が行われたが，その治療成績が科学的に検討されたことはなかった．郭清すると助かるケースもあるという事実から，拡大郭清が正当化されていた状況であった．JCOG の胃癌グループによって大動脈周囲リンパ節郭清の RCT が行われ，標準的な D2 と治療成績に全く差のないことが示されたのはつい最近のことである[2]．

一方オランダでは，D1 郭清と D2 郭清の RCT が行われ，D2 郭清により合併症や手術死亡率は高くなるものの，治療成績の向上は認められなかった[3]．この研究はほとんど RCT のなかった日本の D2 郭清を標準とする主張に，強い疑問を投げかけるものであった．しかし，発表データをみると手術死亡率がきわめて高く，遠隔成績も日本

表1　ステージ別胃癌手術後遠隔成績の比較

報告者	報告年	郭清範囲	症例数	ステージ別5年生存率（%）						
				IA	IB	II	IIIA	IIIB	IV	全体
Wanebo[9]	1993		9,057	59	44	29	15	9	3	26
Siewert[10]	1993	D1	379	86	72	26	25	27	28	
		D2	803	85	68	55	38	17	16	
Pacelli[11]	1993		238	96	73	63	40	33	0	65
Bonenkamp[3]	1999	D1	380	81	60	38	11	13	0	45
		D2	331	81	61	42	28	13	28	47
Degiuli[7]	2004	D2	191	92.5	87.5	60	40	20	2.5	55
日本胃癌学会全国登録	1991年度症例	D2	4,494	93.4	87	68.3	50.1	30.8	16.6	73.7
癌研究会	1994～2004		2,464	94.6	94	79.1	69.9	43.5	14.5	

日本の全国登録のステージは胃癌取扱い規約第12版による.
海外のステージはTNM分類による.

のそれと比較するときわめて不良であったことから，日本においてもD1郭清に留めておくべしという声はほとんど聞かれなかった．D2郭清の経験のほとんどない国で，未成熟な技術をもとに行われたRCTはその国にだけ通用する成果をもたらすのであり，それがそのままほかの国でも受け入れられることにはならなかった．

治療成績

1．短期成績

短期成績を比較するとき，その定義が異なることから術後合併症の発症率に関する単純な比較は難しく，参考程度にしかならない．胃癌の手術死亡率はわが国では時代とともに低下し，最近では1%前後と報告されている．海外における手術死亡率は，主要な臨床試験におけるデータをみてもわが国に比較すると高い（図3）[4~8]．しかし，1960年代に胃癌の根治手術が提唱された時期には，麻酔法や周術期管理が未熟であったこともあり，日本の胃癌手術死亡率は現在に比較すると悪い．その後，外科医が技術向上のために研鑽を重ねるとともに，術後管理技術も進歩し，現在のような低い術後死亡率になったといえよう．特にわが国では胃癌の症例数が多く，外科医が高密度に手術を経験できたことも成績向上につながった．そして，胃癌研究会が結成され，胃癌治療のいわば標準化に向けて熱心な検討が重ねられてきたことも，わが国の胃癌手術技術の向上に大いに貢献した．

2．長期成績（予後）

胃癌手術の長期治療成績も時代とともに改善してきている．たとえば，早期胃癌は現在では95%以上の5年生存率が得られているが，1960年代には80%にすぎなかった．また，進行胃癌の治療成績も年々向上してきたが，近年の治療成績の向上には化学療法もある程度貢献していると考えられる．S-1による術後補助化学療法が，胃癌術後成績の向上に寄与することがRCTで確認された事実もこのような考え方を支持するものである．手術後の遠隔治療成績について国際的に比較しても，わが国の治療成績は抜群によいと考えられる．ステージングも微妙に異なり厳密な比較とはいえないが，同じD2郭清でも日本の治療成績は良好である（表1）[3,7,9~11]．イタリアの一部のグループでは，日本に匹敵する成績が報告されており，熟練した外科医によるD2郭清の重要性が指摘されている[7]．

わが国の手術成績の良好な理由

日本の胃癌の手術成績が良好な理由はいくつか指摘することができるが，第一に，日本において胃癌症例数が多かったことが挙げられる．日常的

に医師がその病態を理解し，治療に当たったその経験を胃癌研究会における検討を通して共有できたことが，胃癌診療のレベルを向上させた第一の要因であると考えられる．この胃癌研究会では外科医だけでなく内科医，放射線科医などの臨床家はもちろん，病理学者が積極的に参加し，胃癌取扱い規約の完成に貢献した．つまり，現代でいうところのトランスレーショナルリサーチが，この時代に実現していたのである．

胃癌取扱い規約という精緻な共通言語のもと，特にリンパ節転移の状況については多数の施設から詳細な検討結果が報告された．その結果，リンパ節の解剖学的な分布とリンパ流に基づいて，合理的なリンパ節郭清範囲，すなわち D2 郭清が提唱された．リンパ節転移の状況がどのようであったのかを一例一例綿密に分析し，そのデータを積み重ねて導き出された結果は，RCT に劣らぬ価値を持つものである．ただし，そのような観察事実に重きを置いたために，D2 郭清が D1 手術より優れているかといったことの検証作業は放置されたままになり，D3 など拡大郭清が検証される結果となった．

しかし，世界各国の手術成績と比較すると，術後の短期成績も長期成績もわが国が格段に優れているという事実は，D2 郭清の有効性と，日本の胃癌手術の技術レベルの高い完成度を示すものと考えられる．また，一律に D2 郭清を行うのではなく，胃癌のほぼ半数を占める早期胃癌に対しては，日本で開発された幽門保存胃切除や迷走神経腹腔枝温存手術など，さらに高度な技術を要求される術式も開発，実用化された．

世界標準は存在するか —「おわりに」にかえて

世界標準治療という言葉が，化学療法の世界ではよく聞かれる．確かに薬剤は製剤が一緒であれば，どの国でも定められた量を同じように投与することが可能である．ただし，薬物代謝の民族差は肺癌における分子標的薬でも明らかである．したがって，いくつかの国で行われた臨床試験の結果をそのまま，すべての国に当てはめることはできない．

一方，外科の技術は，薬物治療と同様それを受ける患者側の要素，つまり肥満度や併存症の有無の頻度などに関して民族差があり，その治療成績は異なることが予想される．また，そればかりでなく手術の質の不均一性が，薬剤より格段に幅広く存在する．その差は，個人差であり施設間差であり，それぞれの国ごとの格差でもある．日本のように国民皆保険制度が整備され，比較的均質な医療技術が提供されている国は，国際的にみてそれほど多くない．米国のように先進諸国でありながら，医療保険制度の不備から大量の無保険者の存在する国はもちろん，最近の社会主義国家でも一部を除けば経済的な問題から，全国民に良質の医療を必ずしも提供できる体制にない．その結果，そのような国で臨床試験を行うにしても，良質な医療提供を受けている富めるものではなく，そもそも医療保険の恩恵を十分受けられない患者が臨床試験に組み入れられている可能性がある．つまり，社会保険制度の違いだけとっても，国際的に均一な試験など施行しようがないのである．

世界標準治療は存在しないといってよいが，国際的な比較をするためには，ステージ分類などは世界標準が必要である．胃癌取扱い規約は日本の胃癌研究の成果の１つであり，世界に誇るべきものであるが，同様の取り扱いを世界にあまねく要求しても到底それは実現しない．本年３月に改定された胃癌取扱い規約が，解剖学的なリンパ節群分類から転移リンパ節個数による分類に変わったことは，今後日本の胃癌診療技術を世界に広めるためにやむをえない措置であった．もちろん，転移個数でステージを決定するからといって，従来から行われているようにリンパ節転移の解剖学的分布も正確に記録しておくことを怠るべきではない．いずれ世界の胃癌治療のレベルが上がってきたときには，再び解剖学的な分布が治療上大きな意味を持つ可能性がある．

胃癌の発生率は減少してきており，ピロリ菌感染による萎縮性胃炎との関係も明らかになりつつある．もし，ピロリ菌感染者に対する除菌が奏効

した場合，わが国でも米国と同様に急激な胃癌症例の減少が見られる可能性がある．そのような状況で日本の優れた胃癌手術がきちんと伝承されるように，今から対策を立てておく必要があるのではないだろうか．手術の RCT は患者に大きな負担を強いるし，その結果が判明し患者に還元されるまでには 10 年近くの年月がかかる．未だ D3 郭清に固執するものもあるが，臨床研究のパワーにも限界がある．このパワーをどのような方向に向けるべきか，真剣に考え直すべき時期にきている．

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(YAMAGUCHI Toshiharu, et al 癌研究会有明病院消化器外科：〒135-8550 東京都江東区有明 3-8-31)

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（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

（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改訂版と初版の公開状況について比較検討しところ，Web公開までの期間は改訂版にて大幅に改善されていた．ガイドライン利用者アンケートでは，ガイドラインの評価は良好であるが各章で評価が若干異なることが判明し，次回改訂時に反映する必要があると考えられた．紙媒体を主として利用されており，今後Web公開の問題点の抽出と改善が必要と考えられた．

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した．

本研究では，このガイドラインの公開体制とガイドライン更新に向けた作業の進め方について検討することを目的とした．

B．研究方法

2009年版肝癌診療ガイドライン改訂後の公開の手順について初版の公開の手順と維持と比較検討し，問題点について考察した．また各HPのアクセスのしやすさや利用頻度になどについて検討するため肝臓学会会員を対象として行われたアンケート調査の結果について検討した．

（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改訂版公開と初版公開の比較について

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センターホームページに公開，その後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ン絵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．冊子出版方公開までの平均期間は平均14ヶ月であった．初版での公開までの期間で短縮がみられたのは，各専門学会での公開が7ヶ月，日本癌治療学会ホームページでの公開が29ヶ月，英語版での公開は40ヶ月の短縮となっていた．

（２）アンケート調査結果について

アンケート回答者は109名，年齢の中央値は46歳，男性93%，女性7%．専門領域は内科系55%，外科系40%，96%以上が卒後10年以上であった．所属診療施設は42%が大学病院，8%が癌専門病院，42%が臨床研修指定病院であった．500症以上の病院が58%，200症以上のび病院が29%であった．診療施設の所在地は東京都が19施設，継いで大阪府10施設，福岡県10施設であった．所属学会では，日本肝臓学会が98%で80%が日本肝臓学会専門医であった．また最近診療した肝癌患者数は外来40人以上，入院20－39人と多く，過去1年間の治療経験数も手術30人以上，局所療法30人以上，TAE30人以上が最も多く，肝癌診療が日常臨床で多い医師が対象であった．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，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をみたことのある率は91%で，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．ガイドラインは日常の診療に役立っていますかという質問には93%で役立っていると回答があった．

ガイドラインが役立たないと回答した理由は，複雑すぎる，日常診療の疑問に答

えていない，であった．ガイドラインに必要なこととして87%が推奨の根拠が明確に示されていること，74%が根拠となっている個々の医学文献の研究内容妥当性が言及してある，と回答があった．37%の医師がガイドラインは医師の裁量を拘束すると考えており，16%の医師が医療費削減のために使われると考えており，32%がガイドラインの普及により医療訴訟が増加すると考えていた．患者用のガイドラインについては70%以上が患者説明や診療の助けになると回答をしていた．86%が医師の生涯教育のために有用であるとし，ガイドライン評価であるAGREE評価表に関しては97%が利用したことがないか認識をしていなかった．EBMに関する認識として80%が日常診療に取り入れており，EBMの考え方は77%が有用であると回答をしていた．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．
ガイドラインにて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．変化は37%が医師の推奨をより強くなった，30%が治療法選択に時間がかからなくなったとの回答であった．各章ごとの評価として分量の多さは平均で90%が適当であると回答し，表現のわかりやすさは平均79.7%が分かりやすいと回答していた．内容の適切さは平均79.9%が適切であると回答していた．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．また重要なエビデンスが出現した場合部分改訂を行うべきかという質問に関しては77%が「はい」という回答であった．66%で医療経済の観点から検討や推奨が必要であると回答をしていた．

D．考察

診療ガイドラインの公開について初版との改訂版での公開までの期間を比較すると改訂版では公開までの期間が平均で27ヶ月から14ヶ月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英語版の公開と日本癌治療学会ホームページでの公開が短縮されたためであった．英語版については冊子公開前に既に英文化を取り込みかつ日本肝臓学会の機関誌であるHepatology Research誌に公開したため短縮が図れたものと考えられ，日本癌治療学会ホームページについては前回ホームページ公開から日時が経過しておらず，公開までの過程が確立されたためと考えられた．

ガイドライン利用者に関するアンケートでは，ガイドラインは診療時に用いられることが多いもののほとんどは紙媒体(出版物)による利用であった．9割の医師はガイドラインが役に立っていると回答し，最も利用されているものはアルゴリズムであった．ガイドラインにより医師の裁量が制限されると感じたり，医療訴訟が増える，医療費削減に用いられるという意見も少なくなかった．またガイドラインの各項目により分量やわかりやすさ，適切さの評価に多少のばらつきはみられたものの妥当であると考えられた．改訂期間については，2年に1度または4年に一度という意見が多く，エビデンスに応じて部分改訂を希望する意見が多かった．

E．結論

肝癌診療ガイドライン改訂版の公開を初版と比較すると公開までの期間の改善がみられた．利用者アンケートの結果からガイドライン自体の評価は良好であるが，紙媒体による利用がほとんどでありホームページ上の公開のあり方についても利用者の立場からさらに検討と改善が必要であると考えられた．また改訂までの期間中の重要なエビデンスについてどのように取り扱うかについても検討する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．

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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 B_{109}	Cyp B	7	KFHRVIKDF	0.5 ± 0.9
5	Cyp 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 $2/neu_8$	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-

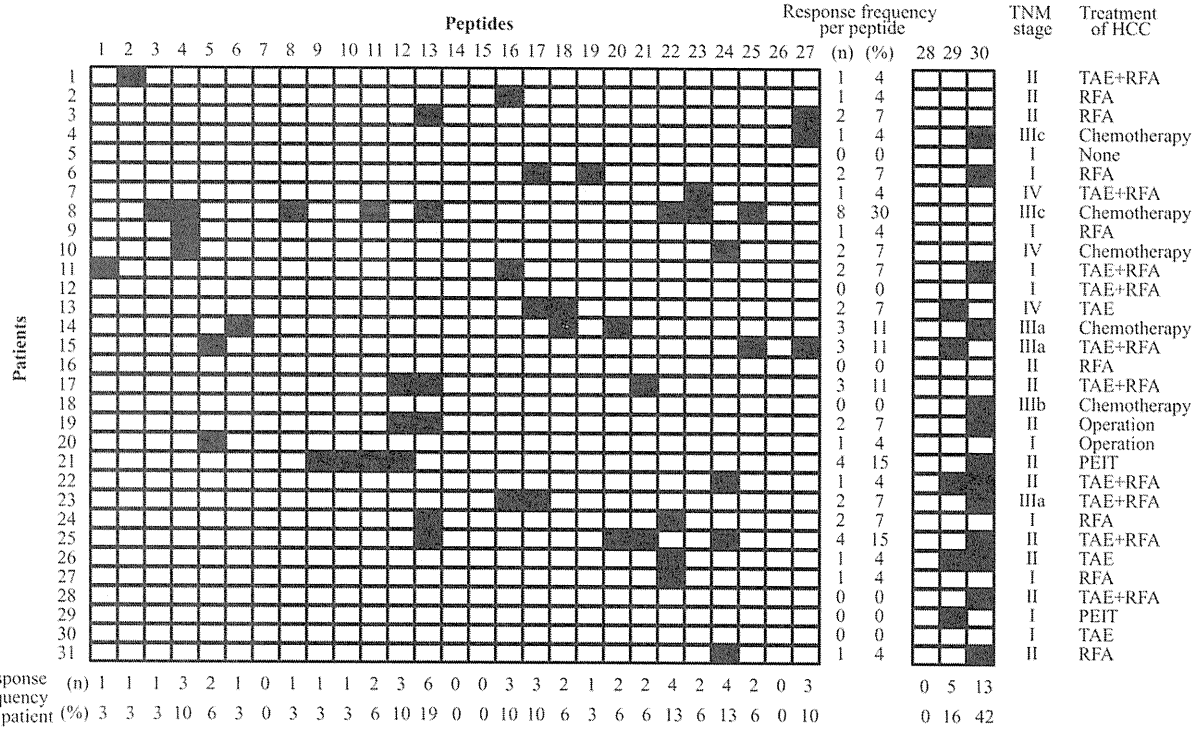

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if 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

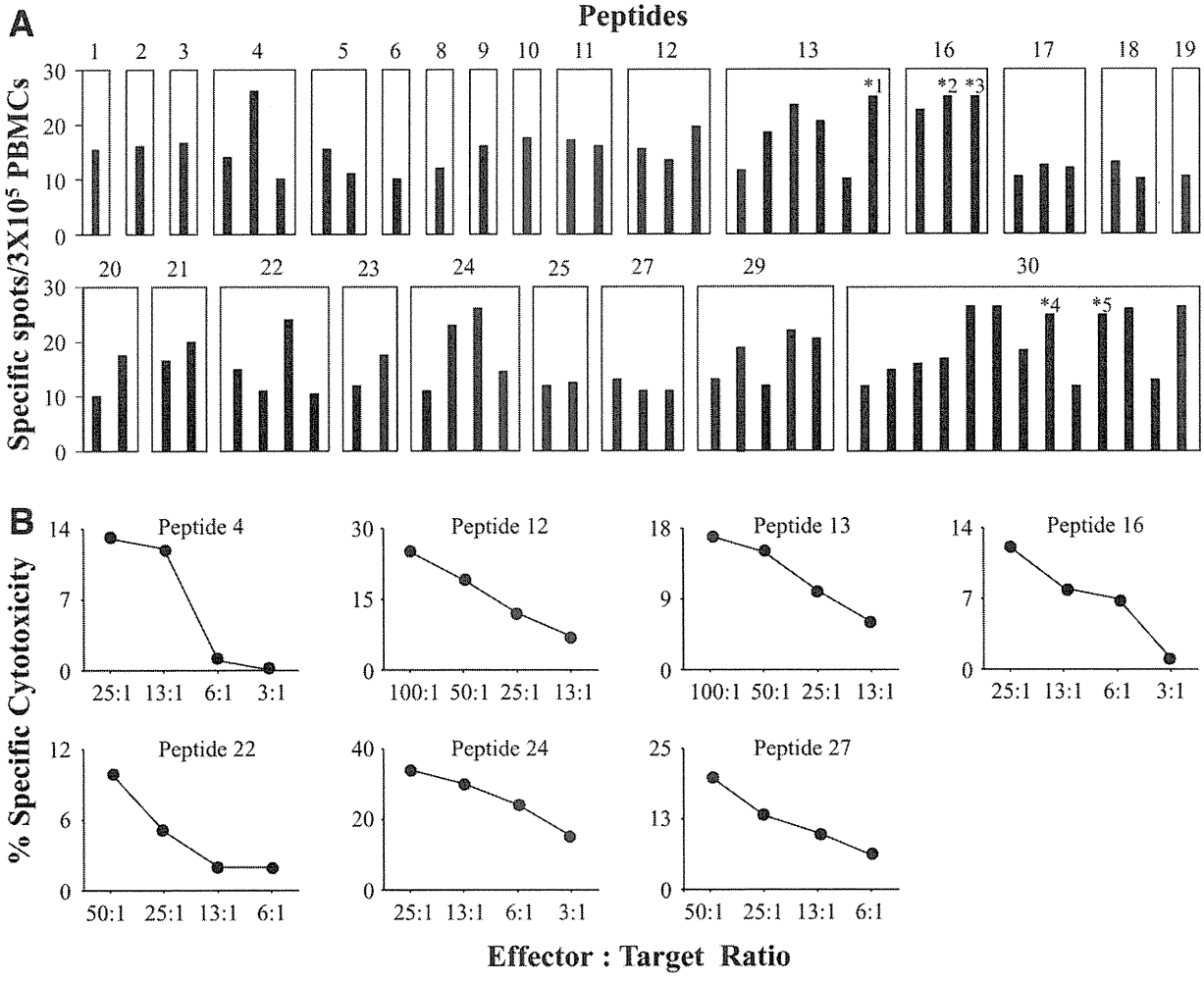

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, 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%)

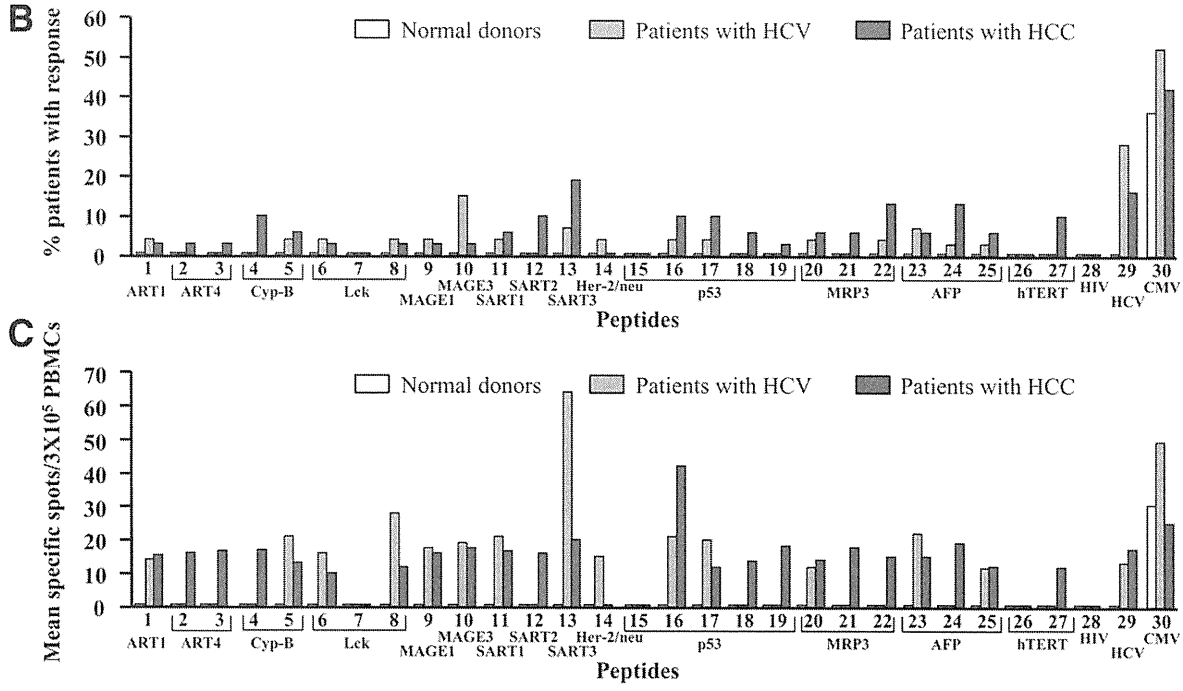

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response 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

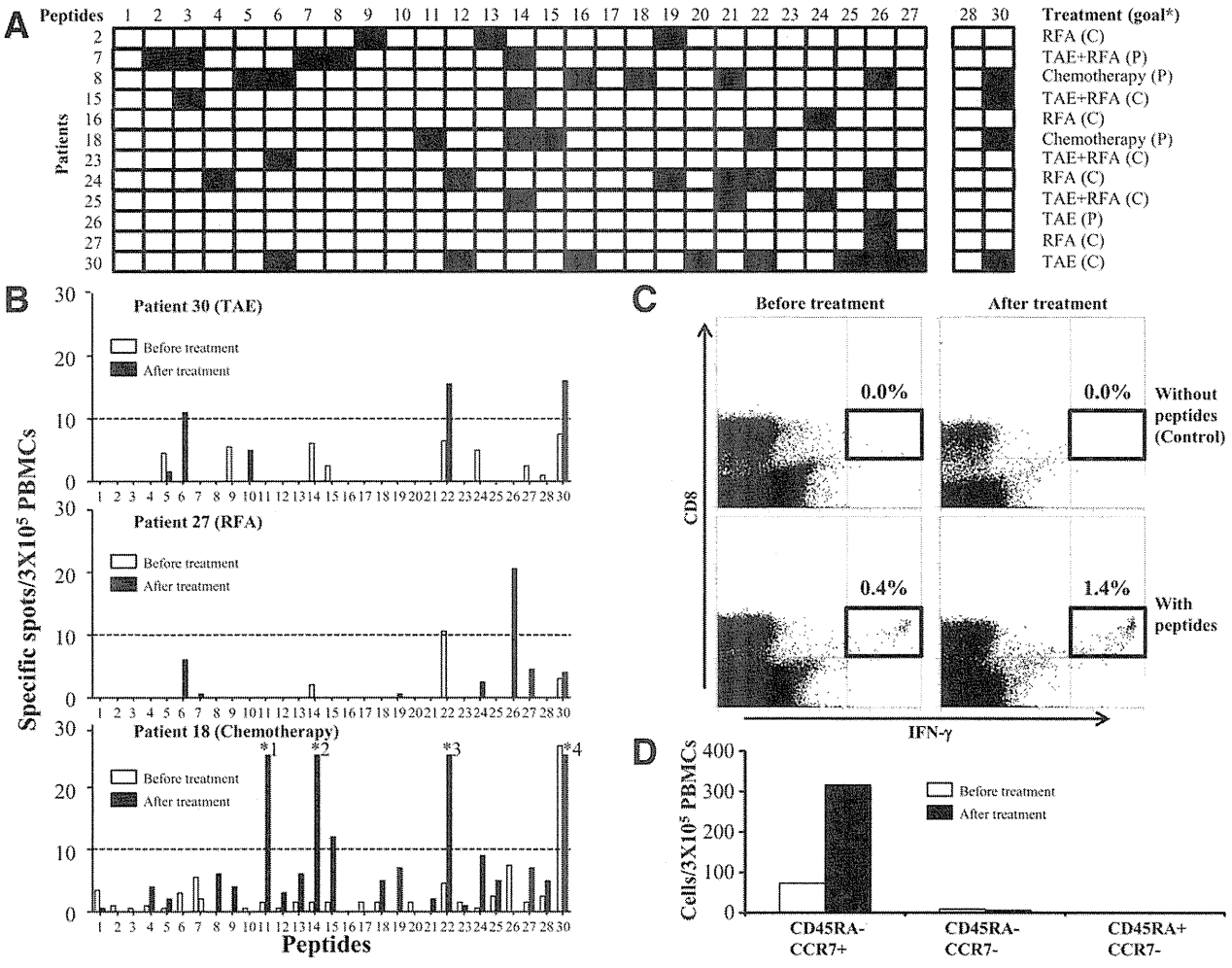

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the 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-

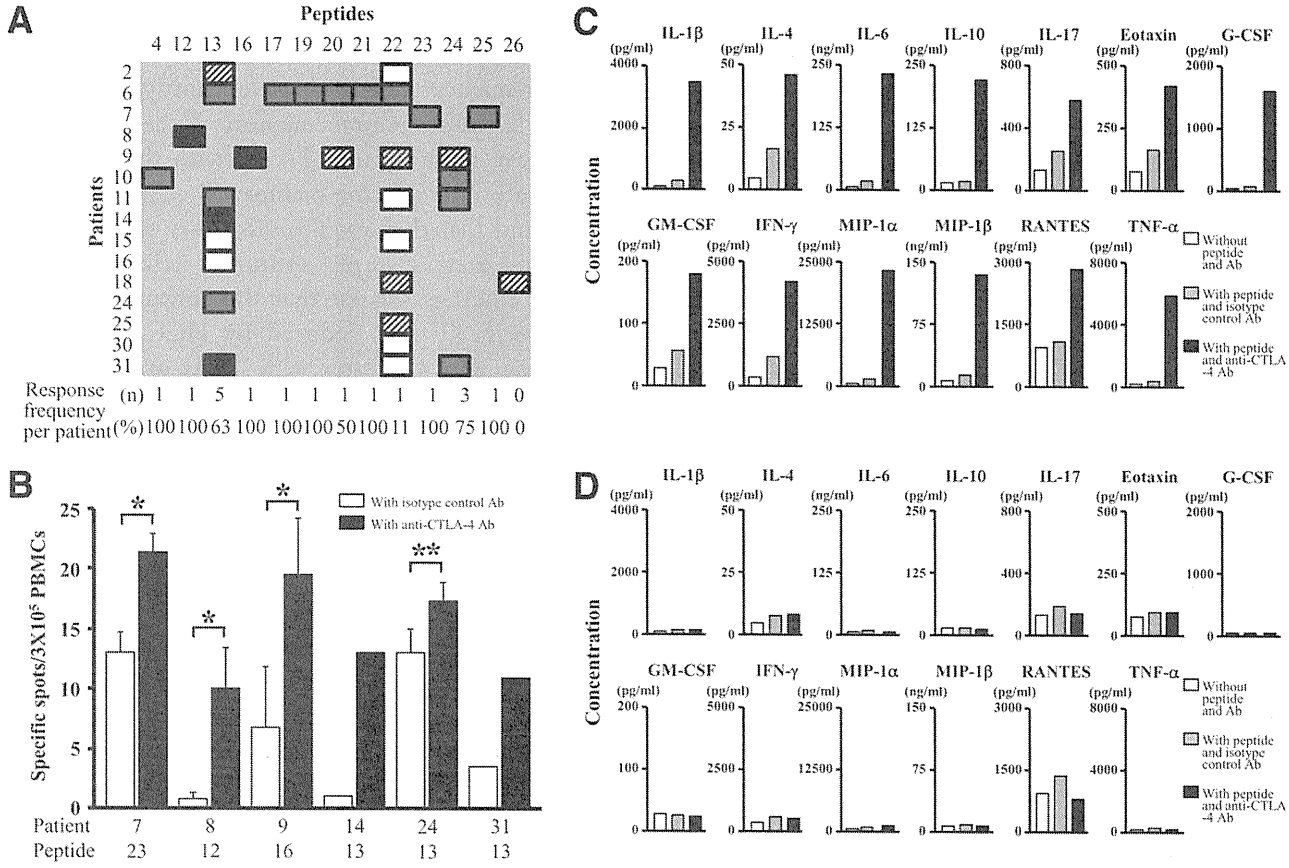

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, 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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Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma

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The identification of genes involved in tumor growth is crucial for the development of inventive anticancer treatments. Here, we have cloned a 17-kDa secretory protein encoded by *c19orf10* from hepatocellular carcinoma (HCC) serial analysis of gene expression libraries. Gene expression analysis indicated that *c19orf10* was overexpressed in approximately two-thirds of HCC tissues compared to the adjacent noncancerous liver tissues, and its expression was significantly positively correlated with that of alpha-fetoprotein (AFP). Overexpression of *c19orf10* enhanced cell proliferation of AFP-negative HLE cells, whereas knockdown of *c19orf10* inhibited cell proliferation of AFP-positive Hep3B and HuH7 cells along with G1 cell cycle arrest. Supplementation of recombinant c19orf10 protein in culture media enhanced cell proliferation in HLE cells, and this effect was abolished by the addition of antibodies developed against c19orf10. Intriguingly, c19orf10 could regulate cell proliferation through the activation of Akt/mitogen-activated protein kinase pathways. Taken together, these data suggest that c19orf10 might be one of the growth factors and potential molecular targets activated in HCC.

Hepatocellular carcinoma (HCC) is one of the most common cancers with an estimated worldwide incidence of 1,000,000 cases per year.[1] Most HCCs develop as a consequence of chronic liver disease such as chronic viral hepatitis due to hepatitis C virus (HCV) or hepatitis B virus (HBV) infection.[2–7] Liver cirrhosis patients with any etiology are considered to be at an extremely high risk for HCC.[8–10] Indeed, ~7% of liver cirrhosis patients with HCV infection develop HCC annually,[8,11] and the advancement of reliable HCC screening methods for high risk patients is crucial for the improvement of their overall survival.[12]

Currently, imaging diagnostic techniques such as ultrasonography, computed tomography, magnetic resonance image and angiography are the gold standards for the early detection of HCC.[13,14] In addition, tumor markers such as alpha fetoprotein (AFP) and des gamma carboxyl prothrombin (DCP) have been used for the screening of HCC,[15–18] although their sensitivity and specificity are not sufficiently high. Recently, a gene expression profiling approach shed new light on Glypican 3, a heparin sulfate proteoglycan anchored to the plasma membrane, as a potential HCC marker, and its clinical usefulness as a molecular target as well as a tumor marker is presently under investigation.[19]

There are several options available for the treatment of HCC, including surgical resection, liver transplantation, radiofrequency ablation, transcatheter arterial chemoembolization and chemotherapy, while taking the HCC stage and liver function into consideration. Recently, molecular therapy targeting the Raf kinase/vascular endothelial growth factor receptor (VEGFR) kinase inhibitor sorafenib improved the survival of patients with advanced HCC,[20,21] emphasizing the importance of deciphering the molecular pathogenesis of HCC for the development of effective treatment options.

Here, we investigated the gene expression profiles of HCC by serial analysis of gene expression (SAGE) to discover a novel gene activated in HCC.[22–25] We identified a gene, *c19orf10*, overexpressed in HCC and determined that the encoded 17 kDa protein (c19orf10) is a secretory protein. Murine *c19orf10* was originally discovered to encode a cytokine interleukin (IL) 25/stroma derived growth factor (SF20) in 2001.[26] The gene *c19orf10* was mapped in the H2 complex region of mouse chromosome 17 between *C3* and *Ir5*, and the hypothetical protein was predicted as globular protein.[26] However, the subsequent study failed to reproduce its proliferative effect on lymphoid cells, and the paper was retracted by the authors in 2003.[26,27] Nevertheless, independent studies revealed that c19orf10 was indeed produced by synoviocytes, macrophages and adipocytes, although the function of c19orf10 remained elusive.[28,29] In our study, we identified that *c19orf10* was overexpressed in AFP positive HCC samples. Our data imply that c19orf10 could activate the mitogen activated protein kinase (MAPK)/Akt pathway and

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