特集　進化するがん免疫療法(ワクチン療法，細胞療法，抗体療法)

ゲノム解析による腫瘍抗原の同定*

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Key Words：tumor-associated antigen, direct immunology approach, reverse immunology approach

はじめに

1991年，Boonらがはじめてヒト悪性黒色腫からがん拒絶抗原を同定[1]して以来，多くの研究者が同様の手法でヒトがん抗原を同定した．キラーT細胞(CTL)クローンを誘導したのち，それが腫瘍細胞mRNAから作製したcDNAライブラリーをもとに遺伝子導入したどの細胞株と反応するかを指標に遺伝子から腫瘍抗原を同定する手法である．今日では直接法(direct immunology)と呼ばれる本法を用いて，多くのがん腫に対して多くの腫瘍抗原が同定された．しかしながら，それらを用いた臨床研究では腫瘍縮小率は全体の2～3％にすぎず[2]，がん治療ワクチンの創薬には大きな失望感が漂った．さらに，CTLクローンを誘導，維持しながら標的細胞との反応性を検討して遺伝子を絞り込む本法は多大な時間と人的労力を要する上に，細胞培養にかかるコストも大きく，新たなブレークスルーが必要であった．その後，網羅的遺伝子解析からがん特異的な発現を示す分子を同定する手法が開発され，新たに有望な腫瘍抗原を同定することに成功している．CTL誘導を目標としながらもCTL反応からの遺伝子同定ではなく，ゲノム全体から適切ながん抗原を絞り込む手法(reverse immunology)はまさに「急がば回れ」の発想といえよう．本稿では，ゲノム解析による腫瘍抗原の同定法についての代表的なこれらの方法につき解説する．

cDNAライブラリー法(direct immunology approach)

1991年，Boonらはメラノーマ(悪性黒色腫)に対するCTLクローンを樹立する一方で，メラノーマ細胞からmRNAを抽出し，cDNAライブラリーを作製した．そして，50～100遺伝子を含むこのライブラリーから得た各遺伝子とMHCクラスI分子を発現させたCOS細胞を標的細胞として先のCTLクローンとの間でみられるキラー反応を測定した(図1)．こうしてCTLクローンが認識する遺伝子由来の腫瘍関連抗原(MAGE-1)を同定することに成功したわけである．その後，メラノーマに限らず，腺がん，扁平上皮がんからも次々と同様の手法で腫瘍関連抗原が同定された．この手法はCTLクローンを誘導し，その反応性から腫瘍抗原を同定する直接的な手法であるためdirect immunology approachと呼ばれる．こうしてヒトがんにおいて腫瘍関連抗原が数多く同定[3)～5)]されたが，この方法は患者末梢血リンパ球からCTLクローンを樹立し，さらにそれを維持させる必要があり，細胞培養にかかる時間と労力は大変なコストである．これを打開するために，次項で述べる網羅的遺伝子解析法による腫瘍関連遺伝

* Identification of tumor-associated antigens by genome analysis.

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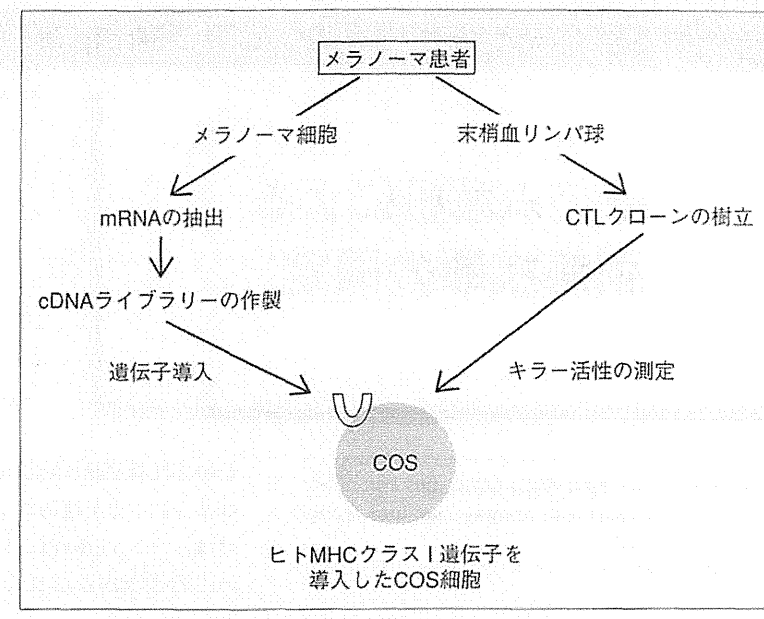

図1　ヒトメラノーマから腫瘍関連抗原(MAGE-1)の同定(direct immunology approach)

表1　網羅的遺伝子解析による腫瘍関連抗原の同定(reverse immunology approach)

段階(ステップ)別目的	方　法	確認事項
1．候補遺伝子の抽出	LMMによるがん細胞，正常細胞の採取 cDNA microarrayによる発現解析	がん細胞，正常細胞の純度 がん高発現遺伝子(群)の抽出
2．候補遺伝子の選別	siRNAによる発現抑制	がん細胞増殖の停止，アポトーシスの誘導
3．ペプチドの結合予測	予測ソフトウェア(BIMAS®)による ペプチドの同定	HLAクラスⅠ分子と高い結合能を有するペプチドの同定
4．CTL反応の測定	^{51}Cr遊離法によるCTL活性の測定	CTL活性の測定(*in vitro*)と抗原特異性の検証

子の同定法が開発されるようになった．

網羅的遺伝子解析法 (reverse immunology approach)

ゲノム全体を検索してがん特異的な遺伝子発現を検索したのち，それらの中から腫瘍抗原として適切な特性を有する遺伝子産物を探索する手法[6][7]である．本法のキーポイントをいくつかのステップに分けて記載する(表1)．

1．候補遺伝子の抽出

(1)LMM法によるがん細胞と正常細胞の採取

がん組織といっても臨床サンプルはがん細胞と間質(血管，間葉系細胞，線維芽細胞，リンパ球，マクロファージ)から形成されている．臨床的にはそれらをも含めた腫瘍塊が「がん」ともいえるが，遺伝子解析ではそれら間質がノイズとなり，真のがん細胞の遺伝子発現評価を困難にする．そこで考案されたのがレーザーマイクロビームマイクロダイセクション(laser microbeam microdissection；LMM)という手法である．すなわち，凍結切片からLMMを用いてがん細胞のみを切り取り，その細胞からRNAを抽出したのち増幅させてがんサンプルを得る．同様に正常細胞もLMMを用いて採取し，RNAを抽出し，正常サンプルを得る．こうすることによってがん細胞にのみ特異的に発現が亢進している遺伝子(群)の抽出が可能になる．

(2)cDNAマイクロアレイ法による発現解析

LMMによって得たがん細胞，正常細胞サンプルを約30,000種以上のcDNAやexpression sequence tag(EST)を搭載したマイクロアレイにアプライして遺伝子発現プロファイルを解析する．

これらを比較することで正常細胞では精巣または胎児期の臓器以外に発現を認めず，腫瘍特異的に発現する遺伝子(群)の同定[8)9)]がなされる．こうして得られた遺伝子由来の腫瘍特異的分子も精巣はHLAを発現していないため，CTLの攻撃は受けず，免疫学的に腫瘍特異的分子としての機能を発揮するわけである．

2．siRNA法による細胞増殖停止の確認

このようにして得られた腫瘍特異的分子の中には腫瘍細胞に発現はするものの細胞増殖，進展に関与しない分子もありうる．そのような場合，発現が消失しても細胞増殖が進み，結果としてがんの進展が抑えられないため，このような分子は腫瘍拒絶抗原として不適切ということになる．そこでsiRNA法で標的遺伝子発現を抑制した場合，細胞周期の停止，アポトーシスの誘導が起こるか否かを確認することが重要になる．こうしてがん細胞に特異的に発現しているだけでなくがん細胞の増殖に重要な分子，換言すればがん拒絶抗原として適切な分子を絞り込むことができるようになる．

3．予測ソフトウェア(BIMAS®)による抗原ペプチドの同定

このようにして得られた新規がん抗原分子から次のステップはCTL誘導を目的とした候補ペプチドの選定である．CTLは標的細胞のクラスⅠ分子上に乗った約9～10個のペプチドを認識することから，本邦では人口の約60％が陽性であるHLA-A*2402に結合するペプチドの同定がよく行われる．もちろんHLA-A*0201,-A*1101などにも結合するペプチドが同定されれば，本邦のほぼ全患者をカバーすることが可能であるが(表2)，まずこのようなアプローチ，すなわちペプチドワクチン療法ががん治療として有効であるか否かを検証することが先決である．具体的には候補遺伝子のアミノ酸配列の中で，あるポジションから開始される9または10アミノ酸配列の候補をもとにそれぞれの結合予測スコアをコンピュータソフトウェアで算出し，スコア値の高いペプチド配列を選定する．

4．CTLの誘導と^{51}Cr遊離法による活性測定

次のステップは，こうして同定された9 merないし10merペプチドによる特異的CTLの誘導能とそのキラー活性強度の測定である．これは，HLA-A24陽性の健常人末梢血リンパ球を用いて^{51}Cr遊離法で測定する．候補ペプチドをパルスした抗原提示細胞で*in vitro*共培養してペプチド反応性CTLを誘導する．そのキラー活性は^{51}Crラベルした当該標的細胞の特異的な細胞傷害活性で測定する．CTLの特異性を評価するためには当該ペプチド抗原を有しない標的細胞，HLA-A24抗原以外の標的細胞などをそれぞれ対照として用いる．

表2　日本人のHLAタイプ

HLA-A	頻度(%)
*2402	61
*0201	20
*1101	18
*3303	16
*0206	16
*3101	15
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(日本組織適合性学会より)

臨床試験による評価

こうして得られたペプチドワクチンは臨床試験によって評価される．医薬品としての市販化には企業主導治験が必須であるが，それまでの早期(第I/II相)試験は医師主導の臨床試験として実施されることも多い．基本的にはその評価は化学療法剤の臨床試験を踏襲するが，がんワクチン特有の薬剤特性，作用機構に適合した基準をつくるべきという考えがあり，2009年に米国Food and Drug Administration(FDA)はガイダンス(draft版)[10)]を発行し，public commentsを求めている．わが国でも日本バイオセラピィ学会が中心になり，がんペプチドワクチンのガイドラインを作成すべく活動を行っている．2011年末には初版を発行できる見込みである．

おわりに

ゲノム解析による腫瘍抗原の同定について2つの代表的な解析法を解説した．cDNAライブラリー法はBoonらによってメラノーマを用いて開発されたが，伊東恭悟教授(久留米大学)らはわが国で頻度の高い腺がん，扁平上皮がんにおいて解析を進め，これまで40～50種類のがん抗原をペプチド

レベルで同定している．彼らは各種の進行がん患者に対してペプチド応答能を測定した上で，投与ペプチドを選択するテーラーメイド型がんワクチン療法を展開している[11]．一方，網羅的遺伝子解析法は中村祐輔教授(東京大学医科学研究所)らを中心に多種類のがん腫において解析され，それぞれに高発現する有望なペプチドが多数同定された．その臨床効果を検証するのはわれわれ臨床医の仕事である．現在は全国約60施設の臨床機関による医療ネットワーク(Captivation Network)が形成され，その臨床効果が検証されている[12]．ペプチドワクチン療法に適切ながん腫は？何種類のペプチドを使うべきか？抗がん剤は併用すべきか？併用するならその抗がん剤は？治療法がなくなった状態の患者を対象とするのは適切か？術後補助療法の適応は？などなど解決すべき問題は山積しているが，年に1度全国の研究者が一堂に会し，進捗状況を報告しながら今後の方向性を模索している．成果は着実に集積しており，一般臨床の現場にがんペプチドワクチンを還元できる日はそう遠くない将来であることを確信しつつ，本稿を終える．

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Stage III 大腸癌におけるがんペプチドワクチンと UFT/LV 併用による術後補助療法（HLA-Key Open 法）

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Preliminary Study of Peptide Vaccine with UFT/LV as Adjuvant Setting for Stage III Colorectal Cancer: Kiyotaka Okuno, Fumiaki Sugiura, Jin-ichi Hida, Tadao Tokoro, Eizaburo Ishimaru and Kazuki Ueda (*Dept. of Surgery, Kinki University Faculty of Medicine*)

Summary

cDNA microarray technology has been used to identify HLA-A24-restricted epitope peptides as potential targets for cancer vaccination in metastatic colorectal cancer patients. We conducted a clinical trial of two novel cancer-specific peptides (RNF43, TOMM34) with UFT/LV for the treatment of recurrent colorectal cancer. Among 23 patients, 21 patients had completed the protocol. All patients were well tolerated with no severe toxicities. The median survival time was 24.4 months. Furthermore, we investigated the relationship between CTL response to both antigens and overall survival. The best long-term survival was observed in the group with CTL responses against both antigens, followed by the group showing CTL responses against only RNF43 or TOMM34. The patients with no response had the lowest survival. Based on the results, we started a randomized trial of the current protocol, as adjuvant immunochemotherapy in following curative resection of Stage III colorectal cancer patients. Key words: Peptide vaccine, Immunochemotherapy, Colorectal cancer

要旨　網羅的遺伝子解析によって同定された大腸癌高発現性遺伝子からいくつかの HLA-A24 拘束性の新規がんペプチド（RNF43, TOMM34）が同定された。23 例の再発・進行大腸癌に対して，がんペプチドワクチンと経口抗癌剤 UFT/LV の併用療法を施行したところ完遂できた 21 例では，有害事象が軽微で，全生存期間中央値（MST）も 24.4 か月と良好であった。しかも RNF43, TOMM34 ともに CTL 反応性がみられた群が最も生存期間が長く，次いでどちらか一方の反応が得られた群，いずれにも反応がなかった群は最も生存期間が不良であった。この結果を基に Stage III 大腸癌の術後再発予防に本療法を応用すべく，ランダム化比較試験を開始している。

緒　言

Stage III 大腸癌の術後補助療法のレジメンはいまだ議論の余地がある。欧米では大規模試験（NSABP C-07 試験，MOSAIC 試験）の結果から oxaliplatin を基本とした強力な化学療法が推奨されるが，手術療法の成績が良好なわが国では副作用の強いその療法を補助療法として用いることに抵抗が強い。われわれはこれまで東京大学医科学研究所，中村祐輔教授らとともに大腸癌に高発現性の遺伝子由来がんペプチドと経口抗癌剤 UFT/LV を極度再発・進行大腸癌に対して投与する臨床試験を行ってきた。その結果，本療法は副作用が軽微で，安全に施行可能であることに加えて，ペプチド抗原特異的キラー T 細胞（CTL）が誘導され，さらにそれが生存期間と相関が得られることを確認した。

CTL は，微小残存癌に有効性が高いことを考えれば本療法こそ Stage III 大腸癌の術後補助療法に最適であると考え，実用化に向けた臨床試験を開始している。

I. 切除不能の再発・進行大腸癌に対するペプチドワクチンと UFT/LV 併用療法[1]

再発・進行大腸癌患者 23 例に対して，網羅的遺伝子解析によって同定された大腸癌高発現性の新規遺伝子 RNF43 と TOMM34 由来の HLA-A24 拘束性ペプチド

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Table 1 Patient characteristics

No.	Age	Gender	Primary care	Sites of Mets	PS	Previous treatment
1	56	M	R	Pelvis	0	UFT, CPT-11
2	64	F	S	Lung	0	5-FU, UFT/LV
3	57	F	R	Lymph nodes	1	5-FU/LV, CPT-11, S-1
4	42	M	R	Pelvis	0	None
5	53	F	S	Lung	0	UFT/LV, vaccine
6	54	M	R	Lung	0	None
7	74	F	S	Lymph nodes	0	5-FU, UFT/LV
8	78	M	R	Lung, lymph nodes	1	5-FU, UFT/LV, CPT-11
9	58	M	R	Lung	1	None
10	46	M	T	Liver, lymph nodes	1	FOLFOX, FOLFIRI, vaccine
11	59	M	S	Primary ca, liver, lymph nodes	1	FOLFIRI, FOLFOX
12	66	M	S	Lung, liver, lymph nodes	0	S-1
13	66	F	RS	Lung	0	UFT/LV
14	49	M	S	Lung, liver	0	None
15	51	F	S	Liver, lymph nodes	1	UFT/LV, CPT-11
16	66	M	R	Lung, liver, lymph nodes	1	UFT/LV
17	61	F	C	Liver, lymph nodes	1	FOLFOX+Bev, FOLFIRI+Bev
18	54	M	S	Primary ca, liver, lymph nodes	0	FOLFOX+Bev, UFT/LV
19	83	M	S	Lung	0	UFT
20	66	M	R	Lung, pelvis, bone	0	FOLFOX+Bev, FOLFIRI+Bev
21	61	M	R	Lung, pelvis	1	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
22	73	M	R	Lung, pelvis, lymph nodes	0	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
23	65	M	R	Lung, pelvis	0	FOLFOX+Bev, FOLFIRI+Bev, IRIS

Bev: bevacizumab, Cet: cetuximab

Table 2 Adverse events

Toxicity	Total	Grade 1	Grade 2	Grade 3
Anemia	5(24%)	5(24%)	0	0
Transaminase elevation	3(14%)	3(14%)	0	0
Hyperbilirubinemia	2(10%)	2(10%)	0	0
Anorexia	5(24%)	5(24%)	0	0
Nausea	2(10%)	2(10%)	0	0
Malaise	3(14%)	3(14%)	0	0
Vaccine site reaction	17(81%)	17(81%)	0	0

と経口抗癌剤UFT/LVを投与した(Table 1)。RNF43, TOMM 34ペプチドワクチン各1 mgをモンタナイド1 mLに溶解して1週間ごとに皮下投与，UFTとUzelは各300 mg/m^2/day，75 mg/dayを4週投与，1週休薬の5週間を1コースとし，2コース繰り返す。各コースの前後で画像評価，安全性評価ならびに末梢血リンパ球採取を行い，それぞれのペプチド特異的CTL反応(ELISPOT assayによるIFN-γ産生能)を測定した。以降は患者希望に応じて治療を延長，継続した。その結果，本プロトコールは重篤な有害事象なく，安全に施行できた(Table 2)。23例中21例が本療法を完遂し，最長例は3年以上を経過している。21例のうちRNF43, TOMM34両方にCTL活性が誘導されたのは8例，どちらか一方のCTL活性が誘導されたのが12例，いずれの活性も認められなかったのは1例であった。全症例の無増悪生存期間(PFS)，全生存期間中央値(MST)はそれぞれ7.2か月，24.4か月であった。さらに，CTL活性の誘導能と生存期間との関連を検討したところ，両方にCTL活性が誘導された群が最も生存期間が良好であり，次いでどちらか一方にCTL活性が得られた群，いずれのCTL活性も得られなかった群(1例)が最も生存期間不良であった(Fig. 1)。

II. Stage III大腸癌の術後再発予防をめざしたUFT/LV併用ペプチドワクチン療法(HLA-key open法)

この免疫化学療法は，担癌量の少ないStage III大腸癌の術後再発予防にこそ威力を発揮できることが予想される。そこでわれわれはStage III大腸癌の術後に本治療を6コース投与するプロトコールを作製し，その安全性ならびに実行可能性(feasibility)を検討した。本ペプチドワクチンはHLA-A24拘束性であるため，HLA-A24陽性症例に有効で，それ以外は無効となるはずである。本邦ではHLA-A24陽性は約60%であるため，理論上，6：4に割り振られることになる。これはHLA-key open

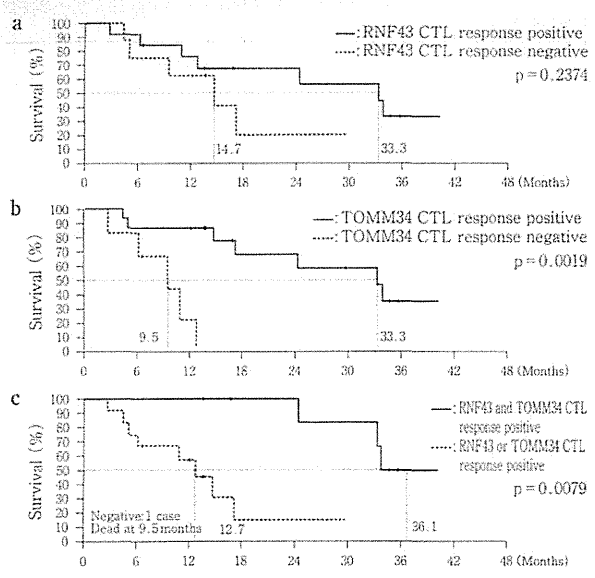

Fig. 1 The relationship between CTL responses and overall survival

a: CTL response to RNF43 and survival.
b: CTL response to TOMM34 and survival.
c: CTL responses to RNF43 and/or TOMM34 and survival.

法というランダム化手法であり，これまでに行われた他の探索的臨床試験でも事実，6：4に割り振られていた。これまで11例に施行し，うち7例はすでに6コースを完遂している。現在はフォローアップ中であるが再発例はなく，今後は多施設共同試験の実施も可能と考えている。

Ⅲ．今後の展望

21例の切除不能再発・進行大腸癌に対するがんペプチドワクチン（RNF43，TOMM34）とUFT/LV併用療法は重篤な副作用がなく，MSTも約2年（24.4か月）と良好であった。しかも2種のペプチド特異的CTL反応性と生存期間に相関がみられたことから，これらがバイオマーカーとして機能する可能性も考えられ，まったく新しいタイプの免疫化学療法として期待される[2]。さらにこの治療法は，Stage Ⅲ大腸癌術後の再発予防にこそ威力を発揮すると考えられる。Stage Ⅲ大腸癌の術後補助療法は，欧米ではoxaliplatin-basedの強力な化学療法が推奨されているが，本来，手術単独の手術成績の良好な本邦では，有害事象の強いそれらを補助療法として利用するには抵抗がある。CTLは，その作用機序から考えても微小癌病巣の治療に適しており，FDAガイダンス（draft guidance）[3]でもそのことが言及されている。それらの観点から本法がStage Ⅲ大腸癌術後の補助療法として応用可能かを検証するため，まずパイロット試験を行ったところ，11例中7例はすでに6コースの治療を安全に完遂できて，そのfeasibilityは確認された。次は探索的研究として少数例での多施設共同ランダム化試験を計画している。もちろん最終的には，製品化をめざして企業主導の治験につなげていく必要があるが，再発予防に勝る治療はなく，医療経済上のメリットも計り知れない。大腸癌の化学療法はすべて欧米の後塵を拝しており，ぜひわが国から新規の免疫化学療法を世界に発信したいと考えている。

謝辞 本研究は東京大学医科学研究所，ヒトゲノム解析センター，中村祐輔教授，角田卓也博士，吉田浩二博士らとの共同研究であり，同研究室には多大なるご協力を賜った。ここに深甚なる謝意を表明する。

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Establishment of a stable T lymphoma cell line transduced with HLA-A*24:02-restricted WT1-specific TCR genes and its application to antigen-specific immunomonitoring

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ABSTRACT

Wilms' tumor gene 1 (WT1) has been proposed as an attractive target for cancer immunotherapy. A natural 9-mer peptide (CYTWNQMNL), which bound to human leukocyte antigen (HLA)-A*24:02, was identified from among WT1-specific cytotoxic T lymphocyte (CTL) epitopes. This natural WT1 CTL epitope peptide was further modified (CMTWNQMNL) to enhance its binding affinity to HLA-A*24:02. This modified WT1 CTL epitope peptide was superior to the natural peptide for inducing HLA-A*24:02-restricted WT1-specific CTLs. Here we induced several WT1 CTLs that reacted with both modified and natural WT1 tetramers from peripheral blood mononuclear cells. Then, T-cell receptor (TCR) genes were isolated from these WT1 CTLs to determine their Vα and Vβ usage. These TCR genes were transduced into human T lymphoma cells to establish a stable cell line, SK37, which expressed a WT1-specific TCR. We confirmed that SK37 cells reacted with both modified and natural WT1 tetramers, which indicated that SK37 cells could be a useful tool for WT1 tetramer reagent quality assurance. One the basis of these findings, we propose that this WT1 tetramer, which was quality-assured using established SK37 cells, will contribute to reliable immunomonitoring of tumor-specific CTL responses of cancer patients who receive WT1-targeted cancer vaccine therapy or TCR-gene therapy.

Cancer vaccine therapy using HLA class I-binding short peptides has been used to induce tumor-specific cytotoxic T lymphocytes (CTLs) in patients with various cancers, because various tumor-associated antigens have been identified in cancer tissues or cells (6, 33). It has been reported that cancer vaccine therapy with short peptides induces increased cancer antigen-specific CTLs and maintains long stable disease in the cancer patients. However, vaccine therapy using short peptides still has numerous issues that need to be resolved for treating cancers (7, 27, 28). Thus, it is necessary to develop more efficient cancer vaccines and establish accurate methods to evaluate cancer antigen-specific immune responses in patients.

It has been reported that Wilms' tumor gene 1 (WT1), which encodes for a zinc finger transcription factor, is overexpressed in cancer cells or tissues of many tumor types, such as acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), and myelodys-

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plastic syndrome (MDS) (2, 10, 13–15, 21, 24, 25). In contrast, WT1 expression is limited in normal cells and tissues of adults (1, 24). Therefore, WT1's product may be a promising specific target for cancer immunotherapy (26). Indeed, a previous report showed that WT1 was the top ranking from among 75 representative cancer antigens on the basis of nine criteria (4).

To date, phase I cancer immunotherapy clinical studies that targeted the WT1 protein have been conducted for AML, MDS, lung, and breast cancer (22, 23, 31) and phase I/II clinical studies using WT1 peptides have been conducted for patients with various types of cancer (29). It was reported that the increase in the numbers of WT1-specific CTLs in cancer patients after vaccination with WT1 peptides closely correlates with the therapeutic efficacy against cancer (7, 16, 17, 20). Therefore, monitoring antitumor immune responses, particularly the generation of tumor-specific CTLs, is critical for accurate assessments of the efficacy of cancer vaccine immunotherapy.

In general, increases in CTLs have been determined by ELISPOT assays, intracellular IFN-γ assays or tetramer assays. The ELISPOT assay, which was developed 18 years ago, has been used by most investigators to determine the frequency of CTL generation in cancer patients after vaccine therapy. It is very easy to determine CTL frequency using this method. However, it is difficult to accurately identify the effector subsets that are responsible for tumor antigen peptide-specific responses with the ELISPOT assay. This is because the possibility that endogenously produced cytokines nonspecifically activate some antigen-nonspecific T cells, NK cells and NKT cells cannot be excluded.

To overcome this problem, human leukocyte antigen (HLA) tetramers have been used to analyze antigen-specific T-cell immunity, because these reagents provide for the accurate enumeration and efficient immunomagnetic sorting of antigen-specific T cells, regardless of the functional capacity of T cells. Therefore, establishing a WT1 tetramer has been suggested to be a very promising tool for immunomonitoring of cancer immunotherapy that uses WT1 peptides.

HLA-A*24:02 (A24) is the major HLA-A allele in approximately 60% of Japanese. Thus, identifying WT1 epitopes for A24 would be important for clinical immunotherapy applications for Japanese cancer patients. An A24-restricted WT1 epitope was previously identified (amino acids 235–243; CMTWNQMNL) (32). In addition, it was demonstrated that a modified 9-mer WT1 epitope (CYTWNQMNL) remarkably increased the binding affinity to A24 molecules and effectively induced WT1-specific CTLs from peripheral blood mononuclear cells (PBMCs) compared with a natural WT1 peptide (A24-natural WT1 peptide). The CTLs that were induced by the modified WT1 peptide (A24-modified WT1 peptide) killed naturally WT1-expressing leukemic cells in an A24-restricted manner. Thus, this modified WT1 peptide is currently being used in clinical studies on cancer immunotherapy using a WT1 peptide vaccine. However, because of the low binding affinity of the A24-natural WT1 peptide to HLA molecules, the A24-natural WT1 tetramer lacks stability compared with the A24-modified WT1 tetramer. Therefore, it was necessary to develop tools that could validate the A24-modified and natural WT1 tetramers. We hypothesized that a stable T lymphoma cell line transduced with A24-restricted WT1-specific T-cell receptor (TCR) genes and had the same binding avidity to both modified and natural WT1-peptide could be a good tool for validating WT1 tetramers.

For this purpose, in the present study, we identified novel WT1-specific TCR genes from A24-modified and natural WT1 tetramer-positive CTLs induced from PBMCs of healthy donors. We used these to establish a TCR gene-transfected T lymphoma cell line, which was designated SK37. Our results suggest that SK37 cells could be used as a positive control in both tetramer-assays and for quality assurance of A24-modified and natural WT1 tetramers.

MATERIALS AND METHODS

Cells, antibodies, tetramers, and flow cytometry analysis. The following cells, antibodies, and tetramers were used for staining and cell sorting. Jurkat, J.RT3-T3.5, and Sup-T1 cell lines were purchased from the Riken Cell Bank (Tsukuba, Japan). CD8-FITC, 7-AAD, and IOTest® Beta Mark were purchased from Beckman Coulter Inc. (Miami, FL, USA). A24-modified WT1 tetramer, A24-natural WT1 tetramer, HLA-A*02:01 Mart-1 tetramer (amino acids 26–35; ELAGIGILTV), and A24-HIV negative tetramer (amino acids 584–592; RYLRDQQLL) were purchased from Medical and Biological Laboratories Co., Ltd (MBL, Nagoya, Japan). Cells were first stained with tetramers at 4°C for 15 min, and then stained with an anti-CD8 antibody at 4°C for 15 min. Flow cytometry analysis used a FACSCalibur (BD Biosciences, San Diego, CA, USA). Data analysis used CellQuest software (BD

Biosciences) and FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

A24-WT1-specific CTL lines. The research protocols for experiments using human specimens were approved by the medical ethics committees of MBL, Institute for Genetic Medicine, Hokkaido University and the Hokkaido University Graduate School of Medicine. Written informed consent was obtained from each subject. WT1-specific CTL lines were established in our laboratory with A24-positive healthy donors PBMCs purchased from Cellular Technology, Ltd. (Cleveland, Ohio) using a mixed lymphocyte peptide culture (MLPC) method as described (11). Cells were grown in complete RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 12.5 mM HEPES, 5% human AB serum, penicillin/streptomycin, 2 mM L-glutamine (referred to as T-cell medium), and 50 U/mL of human recombinant IL-2 (Shionogi Pharmaceutical Institute Co., Osaka, Japan). The CTL lines were periodically stimulated in the presence of irradiated and peptide-pulsed HLA-A*24:02-positive Epstein-Barr virus-transformed B cells (lymphoblastoid cell lines; LCLs).

Positive selection of antigen-specific T cells. CTL lines were stained with A24-modified WT1 tetramer-PE at 4°C for 15 min. After washing with MACS buffer (phosphate-buffered saline supplemented with 0.5% human serum albumin and 2 mM EDTA), the cells were incubated with anti-PE microbeads (Miltenyi Biotech, Auburn, CA, USA) at 4°C for 15 min. AutoMACS (Miltenyi Biotech) was used to prepare separated cells.

Repertoire analysis of TCR β chains. An IOTest Beta Mark® TCR Vβ Repertoire kit was used for the analysis of TCR β chains with antibodies against the following TCR Vβ regions: Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23.

PCR cloning and sequencing of WT1 peptide-specific TCR α/β genes. The TCR α chain composes a TCR alpha chain variable region (TRAV), a joining region (TRAJ), and a constant region (TRAC). The TCR β chain composes a TCR beta chain variable region (TRBV), a diversity region (TRBD), a joining region (TRBJ), and a constant region (TRBC). Total RNA from sorted CTLs was prepared with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and an aliquot (1 μg) was subjected to reverse transcription using an oligo (dT) primer and SuperScript III (Invitrogen, CA, USA). First strand cDNA was amplified by PCR using a FastStart High Fidelity PCR System (Roche Diagnostics, Rotkreuz, Switzerland) with coding region-specific primers for TRBV5-1 and TRBC1/TRBC2 (WT1 peptide specific TCR β chain), various TRAV primers, and TRAC (TCR α chain) according to the manufacturer's instructions. These PCR products were separated on a 1% agarose gel. A band of the appropriate size (bp) was excised and extracted from the gel. The recovered DNA fragment was cloned into the vectors pCDNA3.1 and pEF6/Myc-His (Invitrogen, CA, USA), and its DNA sequence was determined using BigDye Terminator reagent and a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). The confirmed cDNA sequences of each TCR gene were analyzed using the WEB tool IMGT (8). HLA-A*02:01-restricted Mart-1-specific TCR α/β cDNAs (5) were purchased from IDT (Coralville, IA, USA).

Transduction of TCR genes in lymphoma cells. To establish stable transfectants, TCR α chain/pCDNA3.1 and TCR β chain/pEF6/Myc-His plasmids (10 μg each) were transduced into lymphoma cells by electroporation, and selection was done in a medium containing 0.5 mg/mL of G418 (Roche) and 5 μg/mL of Blastisidin (Invitrogen). Expression levels of the transduced genes were assessed by flow cytometry with tetramer staining.

In vitro cytotoxicity assay. A24-positive and A24-negative LCLs in 100 μL of complete medium were labeled with 3.7 MBq ^{51}Cr for 1 h at 37°C. For peptide reconstitution assays, 1 μM of a synthetic peptide was added 1 h before introducing effector cells. After 4 h of incubation with effector cells, supernatants were determined with a gamma counter. The percentage specific lysis was determined by: [(experimental release − spontaneous release) / (maximum release − spontaneous release)] × 100.

Statistical analysis. Results are given as means and standard deviations. Statistical comparisons were made using two-tailed Student's t-tests; *P*-values of 0.05 were considered significant.

RESULTS

Induction of A24-WT1-specific CTL lines by MLPC

We first attempted to establish WT1-specific CTLs to identify TCR genes that bound to the WT1 peptide-A24 complex. WT1-specific CTLs were induced

using PBMCs from A24-positive healthy donors with A24-modified WT1 peptide (Fig. 1A). After repeated stimulation in MLPC, we established seven WT1-specific CTL lines (data not shown). As shown in Fig. 1B, one CTL line (37F8) was recognized by both A24-modified and natural WT1 tetramers. Although both tetramers reacted with 37F8 cells, their reactivity with the A24-WT1 natural tetramer was lower than that with the A24-WT1 modified tetramer. The frequency and absolute numbers of tetramer-positive T cells among 37F8 cells were much higher than those among the other CTL lines (data not shown). We confirmed that A24-WT1-specific 37F8 cells could be successfully purified using an autoMACS separation system with PE-A24-WT1 modified tetramer, and anti-PE microbeads (data not shown). These results suggested that the established 37F8 cells expressed TCRs that could bind to both A24-modified and natural WT1 peptide-HLA complexes.

Identifying the WT1 specific TCR Vβ repertoire

Next, we sought to identify the TCR Vβ repertoire of the 37F8 cells using a TCR Vβ Repertoire Kit, which could account for about 70% of the variations in TCR Vβ. We confirmed that the TCR β chains of 37F8 cells were recognized by anti-TCR Vβ5.1 (Fig. 2).

TCR cloning of the WT1-specific 37F8 cells

We investigated whole TCR sequences expressed by the 37F8 cells sorted by the AutoMACS system with A24-modified WT1 tetramer. TCR α and β genes were amplified by PCR with coding region-specific primer pairs for TRAC and various TCR α chains, or TRBV5-1 and TRBC1/2. PCR conditions were decided upon using Jurkat cells cDNA (TRAV8-4/TRAJ3/TRAC, TRBV12-3/TRBJ1-2/TRBC1) and human PBMCs cDNA that contained many different TCR genes (data not shown). As a result, we found that the TCR Vα chains of the 37F8 cells comprised TRAV12-2, TRAV12-3, and

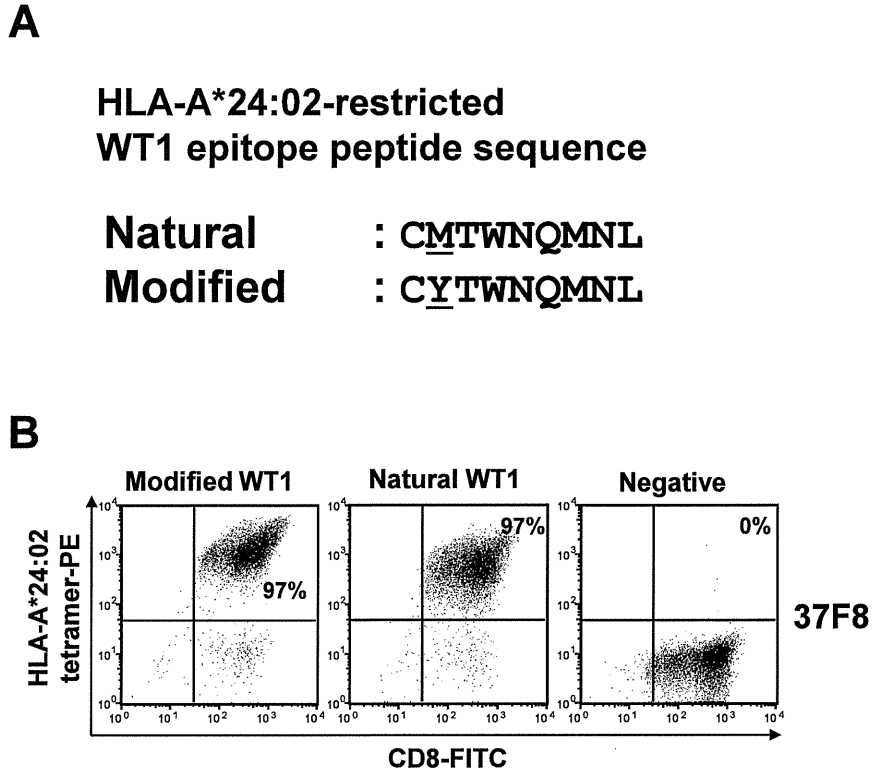

Fig. 1 Establishing WT1-specific CTL lines from PBMCs by MLPC. Upper panel (**A**) shows the CTL epitopes of A24-modified and natural WT1 tetramers used in this study. Lower panel (**B**) shows representative results for WT1-specific CTLs (37F8) induced by MLPC with A24-modified or natural WT1 peptides. The 37F8 cells were stained with A24-modified or natural WT1 tetramers. HIV tetramer was used as a negative control. The percentages of tetramer$^+$ cells among CD8$^+$ T cells are indicated.

TRAV41 (Fig. 3A). Because of high homology, the sequence for the TRAV12-2 PCR product was the same as for the TRBV12-3 PCR product, and the sequence for the TRBV5-1/TRBC1 PCR product was the same as for TRBV5-1/TRBC2. On the basis of the sequence analyses of these TCR α and β PCR products, we concluded that the 37F8 cells had two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and one β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B).

Establishing a T lymphoma cell line that expressed WT1-specific TCRs

To evaluate TCR reactivity with A24-modified and natural WT1 tetramers, we transduced the TCR α/β genes from the WT1-specific 37F8 cells into a Sup-T1 T lymphoma cell line by electroporation with expression vectors. Successful transduction of TCR genes (A12-3 and B5-1) into Sup-T1 T lymphoma cells was confirmed by staining with PE-WT1 tetramers (Fig. 4A). However, the A41 and B5-1 gene-transduced cells did not react with either the A24-modified or natural WT1 tetramers. HLA-A*02:01 Mart-1 TCR was used as a positive control for electroporation and tetramer staining. A stable T lymphoma cell line, SK37, was established by drug selection after transducing the 37F8 TCR A12-3 and B5-1 genes. The reactivity of TCRs expressed on SK37 cells was evaluated by flow cytometry with A24-modified or natural WT1 tetramers. This confirmed that the established SK37 cells reacted with both A24-modified and natural WT1 tetramers whereas an A24-HIV-negative control tetramer did not react with SK37 cells (Fig. 4B).

Functional properties of SK37 TCRs from the 37F8 cells

To investigate SK37 TCR function, cytotoxicity of the 37F8 cells was determined by a ^{51}Cr-release assay. The 37F8 cells showed robust, specific cytotoxicity against A24-positive LCLs that were pulsed with A24-modified or natural WT1 peptides (Fig. 5A and 5B). However, the 37F8 cells did not react with peptide-non-pulsed A24-positive LCLs and A24-negative LCLs (Fig. 5B). These results strongly suggested that the transduced WT1-specific TCR α/β genes could recognize the A24-WT1 epitope peptide.

DISCUSSION

To develop better cancer treatments, we established an accurate immunomonitoring system that could be used to demonstrate the mechanisms of antitumor effects of cancer immunotherapy. An HLA-tetramer reagent is one of the important tools used for immunomonitoring to detect antigen-specific CTLs. It was reported that a tetramer assay using peripheral blood

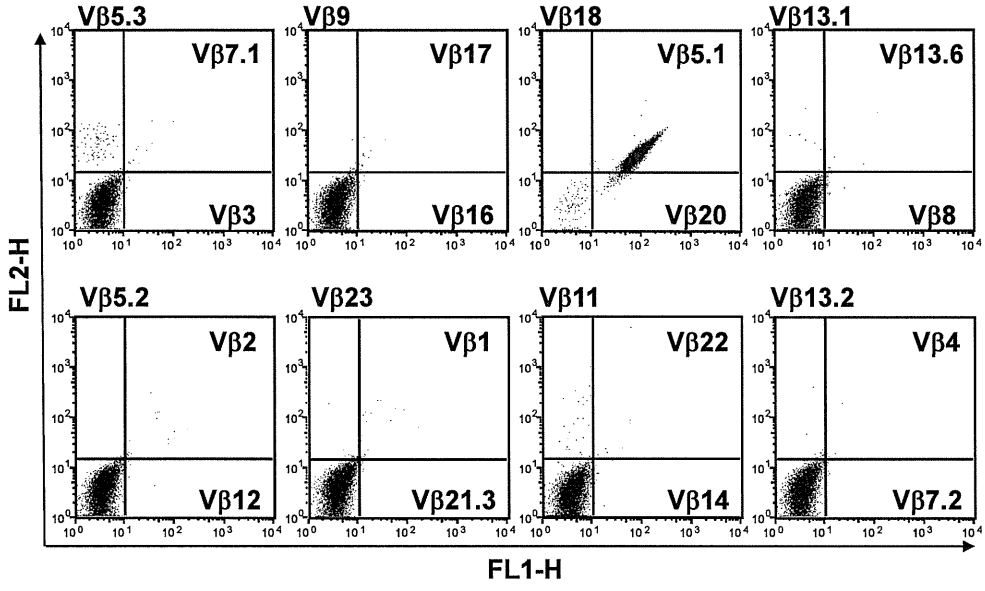

Fig. 2 Analysis of the TCR Vβ chain for the WT1-specific 37F8 cells. 37F8 cells induced by A24-modified WT1 peptide were stained with a variety of anti-TCR antibodies using a TCR Vβ Repertoire Kit. We confirmed that most CTLs reacted with anti-TCR Vβ 5.1. Representative results are shown in this figure.

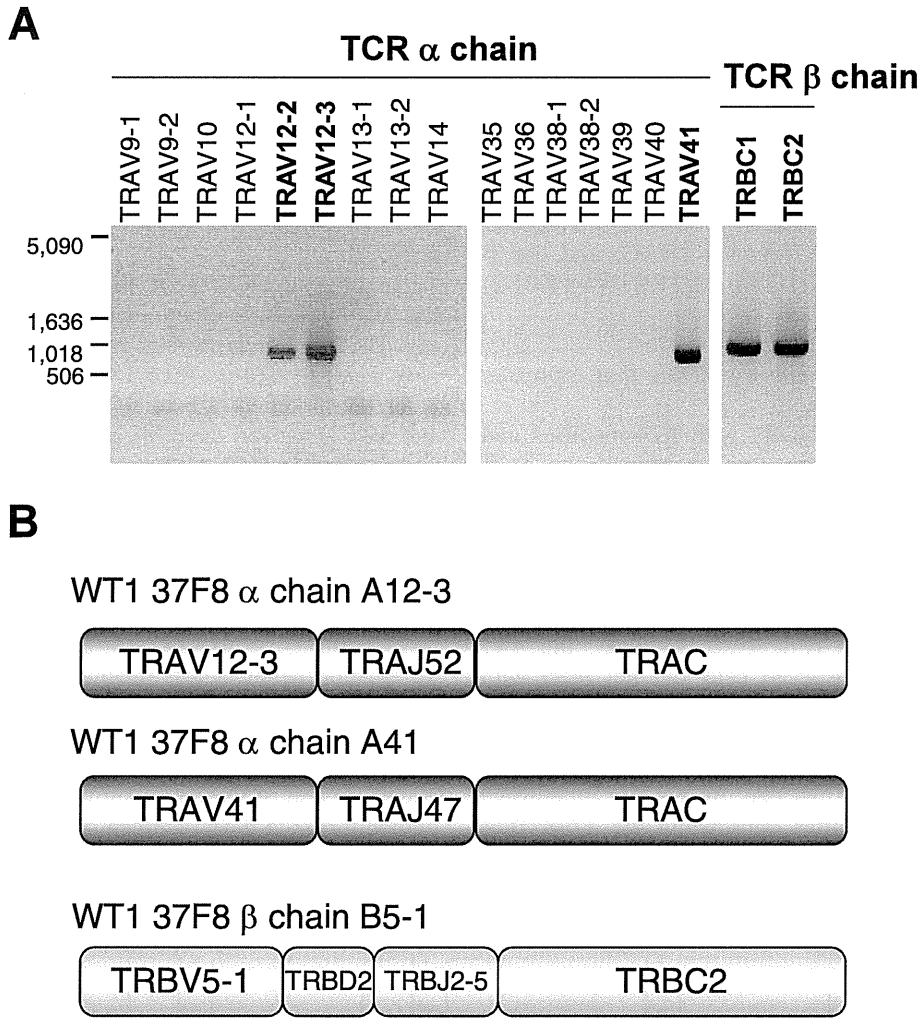

Fig. 3 Identifying the TCR Vα and Vβ repertoires for the 37F8 cells purified with A24-modified WT1 tetramer. TCR α and β genes were amplified with a thermal cycler with PCR coding region-specific primer pairs for TRAC and various TCR α chains, or TRBV5-1, and TCRBC1/2. (**A**) TCR α and β PCR products were separated on agarose gels and visualized by UV illumination. TRAV12-2, TRAV12-3, and TRAV41 genes were detected using PCR with a TCR α primer-set. (**B**) Illustration of TCR α and β chains of the WT1-specific 37F8 cells. The 37F8 cells had two types of α chains (A12-3, A41) and one β chain (B5-1).

from patients could become the best screening procedure, because it could be performed more easily and quickly than conventional procedures (16). However, the frequency of tetramer-positive $CD8^+$ T cells is generally not so high, and it is well-known that they have highly diverse reactivity using current methods. In addition, there is a difficulty with stable quality assurance of tetramer reactivity, because it depends on the specific reactivity between CTLs and HLA-peptide complexes. Therefore, for definitive analysis of antigen-specific immune responses using a tetramer assay, it is necessary to establish a stable cell line that expresses antigen-specific and monoclonal TCR α/β. In this study, we established a lymphoma cell line, SK37, that expressed WT1-specific TCRs, and this cell line could bind to both A24-modified and natural WT1 tetramers (Fig. 4B). These results suggest that SK37 cells could be useful as a positive control to evaluate the quality assurance of A24-modified and natural WT1 tetramers during immunomonitoring.

We successfully identified novel TCR genes composing two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and

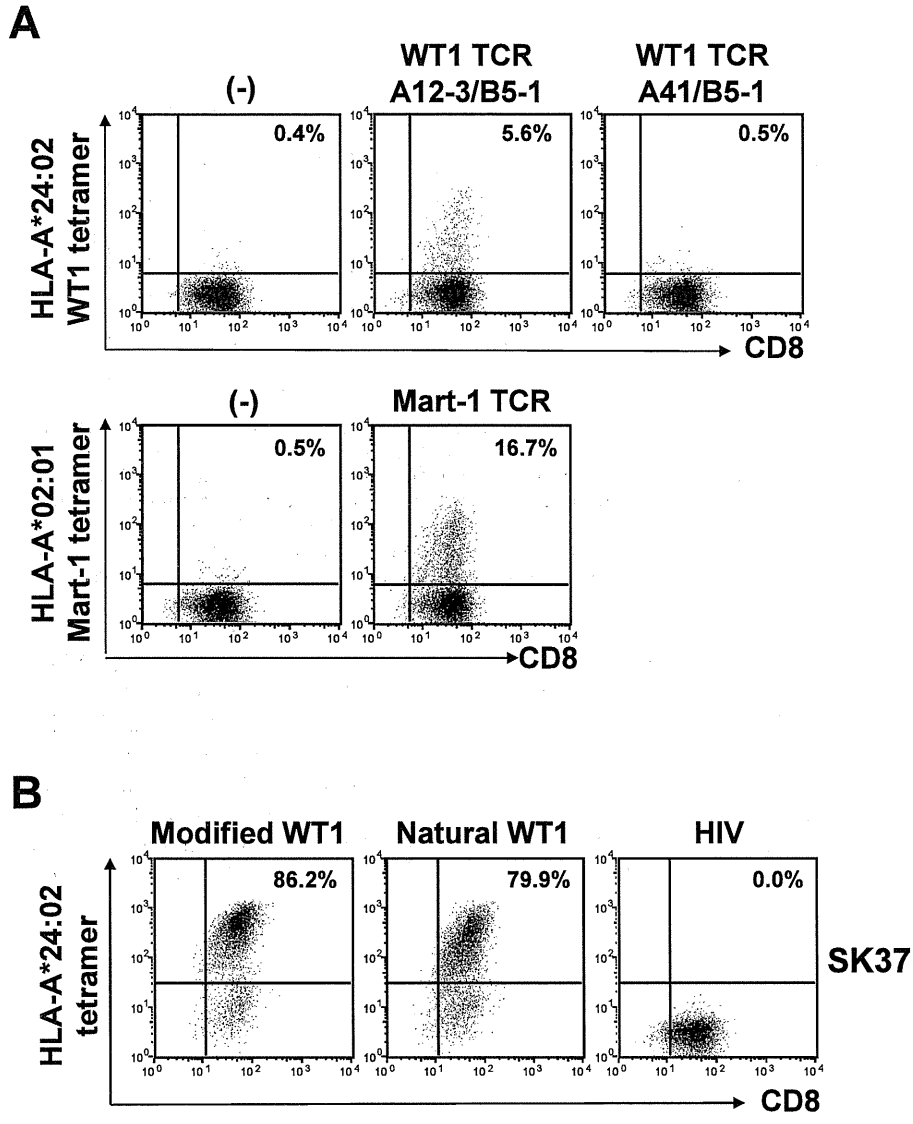

Fig. 4 HLA-tetramer assay for T lymphoma transduced TCR α and β genes from the 37F8 cells. (**A**) WT1 TCR (A12-3/B5-1 or A41/B5-1) genes were transduced into Sup-T1 cells. TCR-transduced T lymphoma cells were stained with A24-modified WT1 tetramer. The A24-WT1 tetramer bound to T lymphoma cells with WT1 TCRs composing A12-3/B5-1 but not with WT1 TCRs of A41/B5-1. A T lymphoma cell line with HLA-A*02:01 Mart-1 TCRs was used as a positive control for electroporation and the subsequent assay with Mart-1 tetramer. (**B**) The SK37 cells were established from T lymphoma cells with WT1 TCRs composing A12-3/B5-1. The TCR reactivity of SK37 cells was evaluated by flow cytometry using A24-modified WT1 (plot at left) or natural WT1 (center plot) tetramer in addition to HIV tetramer as a negative control (plot at right).

a single β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B) for the WT1-specific 37F8 cells that were established using PBMCs from healthy donors by MLPC (Fig. 1B). It was also important to confirm the various functions of the identified TCR genes that recognized HLA/WT1-peptide complexes. We assessed the cytotoxicity of the established CTLs with A24-restricted WT1 TCRs using a ^{51}Cr release assay. In these experiments, the novel WT1 TCR-transduced CTL line showed specific cytotoxicity against natural WT1 peptide-pulsed A24-positive LCLs (Fig. 5A), although their cytotoxicity against modified WT1 peptide-pulsed LCLs was about four times stronger than natural WT1 peptide-pulsed LCLs. We speculated that the differences in cytotoxicity derived from the different affinities be-

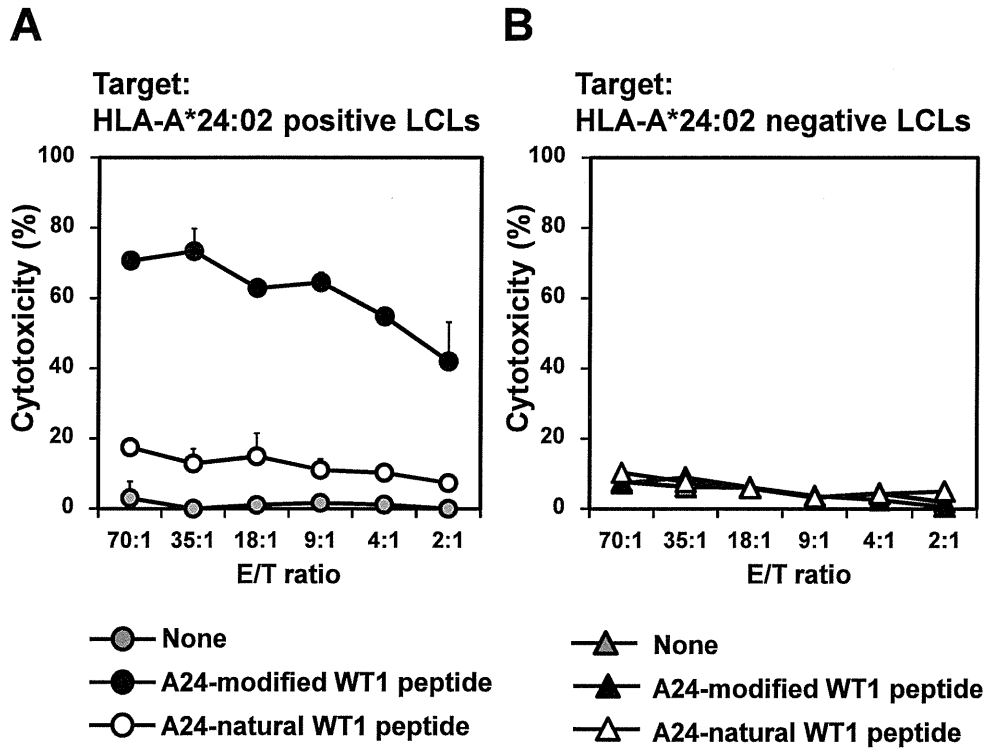

Fig. 5 Cytotoxicity of the WT1-specific 37F8 cells against A24-positive or A24-negative LCLs. A24 positive or A24-negative LCLs, used as target cells, were pre-incubated in the absence (None) or presence of A24-modified or natural WT1 peptides for 1 h, after which 37F8 cells were added as effector cells. CTLs cytotoxicity at the indicated E:T cell ratios was evaluated by 4 h ^{51}Cr-release assay. The cytotoxicity against A24-positive (**A**) and A24-negative (**B**) LCLs are indicated in this figure. 37F8 cells efficiently killed A24-positive target cells pulsed with A24-modified WT1 peptides and also killed fewer target cells pulsed with A24-natural WT1 peptides.

tween HLA molecules and natural or modified WT1 epitope peptides. However, the present findings clearly indicated that the novel WT1-specific TCR-transduced CTLs could recognize naturally processed WT1 peptides and kill endogenously WT1-expressing cancer cells in a WT1-specific A24-restricted manner. Thus, this may become a promising tool for developing TCR gene immunotherapy.

We recently demonstrated that introducing Th1-dominant immunity is essential for inducing fully activated CTLs and immunological memory in tumor-bearing hosts (9, 18, 19, 30). It has also been demonstrated that a mixture of various synthetic long peptides derived from naturally occurring sequences of HPV-16 oncoproteins was superior to short tumor peptides in terms of inducing complete or partial response in vulvar intraepithelial neoplasia (12). Thus, a long peptide vaccine that contains both helper and killer epitopes appears to be a rational strategy to activate Th1-dependent antitumor immunity (18).

As an innovative cancer vaccine, we also developed an artificially synthesized long peptide, H/K-HELP (helper/killer-hybrid epitope long peptide), which was conjugated to a MAGE-A4 class I-binding epitope and our defined helper epitope (3), and used this for a patient with pulmonary metastatic colon cancer. We found that cancer-specific Th1/Tc1 cells were induced in this cancer patient after vaccination with MAGE-A4-H/K-HELP. Therefore, we are now preparing HLA-class II tetramers for accurate immunomonitoring of MAGE-A4- and Survivin-H/K-HELP cancer vaccine therapy.

In summary, we established WT1-specific CTL lines using PBMCs from healthy donors by MLPC. We cloned TCR genes from these CTLs, which were transduced into T lymphoma cell lines by electroporation. As a result, we successfully established a novel A24-WT1 tetramer-positive lymphoma cell line, designated SK37, which was useful for the quality assurance of A24-modified and natural WT1 tetramers for immunomonitoring. Thus, the present

WT1 tetramer reagent that was validated using the SK37 cells could become a diagnostic product for cancer patients' antigen-specific immunotherapy.

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Recent Advances in Active Specific Cancer Vaccine Treatment for Colorectal Cancer

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Abstract: Cloning techniques to identify genes and peptides of tumor-associated antigens have created new possibilities for the immunotherapy of patients with advanced cancer. Here, we review recent clinical trials of specific cancer vaccines, mainly HLA-restricted peptides, and epitope-encoding vectors for advanced colorectal cancer (CRC). Many researchers initially focused on carcinoembryonic antigen (CEA) as an immunologic target antigen that is overexpressed on virtually all CRCs. A recombinant vaccine containing the CEA gene and dendritic cells (DCs) loaded with CEA peptide was administered to patients with CEA-elevated CRC. Although CEA-specific responses were detected, the clinical responses were limited. Recently, new types of clinical trials—namely, a personalized protocol to take into account the immunological diversity of cytotoxic T cell responses among patients and a novel cancer-testis antigen protocol that uses multiple peptides derived from genes identified by the cDNA array method—have been introduced. The personalized protocol seemed to be better than the classical (non-personalized) protocol in terms of clinical response and survival. Novel cancer-testis antigen protocols that use multiple CRC-derived peptides were recently conducted in patients with advanced CRC. The preliminary study yielded promising results regarding specific T cell responses to peptides and survival benefits. In this review, we summarize these results and discuss future perspectives.

Keywords: Active specific cancer vaccine, cancer-specific peptide vaccine, colorectal cancer (CRC), personalized peptide vaccine.

INTRODUCTION

Despite advances in treatment modalities, colorectal cancer (CRC) is still a leading cause of cancer-related mortality in industrialized countries. Improved treatment is urgently needed. Since the discovery of tumor-associated antigens during the early 1990s [1], rapid progress has been made in identifying antigens and describing immune interactions in cancer patients. Immunotherapeutic approaches have entered the clinical phase [2]. The goal of active specific immunotherapy is to induce an *in vivo* tumor-directed immune response. Thus, active specific immunotherapy must be distinguished from passive immunotherapy and nonspecific immunotherapy including cytokines or immunostimulants.

RATIONALE OF IMMUNOTHERAPY FOR COLORECTAL CANCER

The survival advantage of pronounced lymphocytic infiltration in CRC has been known for many years. The pioneering study by Jass showed the improved survival of CRC patients when prominent lymphocytic infiltrate was present [3]. Improved survival in patients with an increased number of peritumoral and stromal tumor-infiltrating lymphocytes (TILs) was demonstrated by Ropponen *et al.* [4]. However, in studies by Nanni *et al.* [5], Nielsen *et al.* [6], and Roncucci *et al.* [7], the number of TILs at the tumor margin or in the stroma did not influence survival in multivariate analysis. Notably, these authors did not investigate the role of intraepithelial lymphocytes (IELs). When Naito *et al.* [8] examined the role of TIL location in relation to prognosis, they found that stromal and peritumoral lymphocytes had no influence on survival, whereas the presence of IELs and CD8+ T cells in cancer cell nests was a predictor of improved outcome, independent of stage. In the same fashion, Funada *et al.* [9] demonstrated that patients with a high level of macrophage and CD8+ T cell invasion at the invasive margin had a 5-year overall survival rate of 92%, compared with a 72% survival rate in patients with a low level of infiltration Table (1).

These contradictory results may result from the complex interactions between lymphocytes, tumor, and microenvironment. It is clear, however, that the presence of activated CD8+ T lymphocytes in cancer cell nests suggests that the lymphocytes are recognizing a tumor antigen, resulting in a better prognosis.

Approximately 15% of sporadic CRCs and most hereditary nonpolyposis CRCs (HNPCCs) exhibit microsatellite instability (MSI) caused by a defect in the DNA mismatch repair system. CRCs with high MSI are usually proximal, poorly differentiated, and associated with pronounced lymphocyte infiltrate, and they have a better prognosis in comparison with MSI-negative tumors [10]. The increased im-

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Table 1. Tumor-Infiltrating Lymphocytes and Survival in CRC

Investigator	Pts N	TIL Location	RFS	OS	Follow-up
Roncucci [7] (1996)	397	Tumor margin	NR	Rectal cancer: 62% vs 36% Colon cancer: 61% vs 54%	5 Years
Ropponen [4] (1997)	195	Margin, stroma	HR, 0.72 (P<0.05)	HR, 0.55 (P<0.05)	14 Years
Naito [8] (1998)	131	Margin, stroma, IEL	NR	HR,0.91 (P=NS), HR,0.81 (N=NS), HR, 0.54 (P<0.05)	5 Years
Nielsen [6] (1999)	584	Tumor margin	NR	HR, 0.66 (P=0.03)	5 Years
Nanni [5] (2002)	263	Stroma	65% vs 58% (P=0.2)	81% vs 72% (P=0.09)	4 Years
Funada [9] (2003)	97	Margin, CD8+T cells	NR	92% vs 72% (P<0.05)	5 Years

Studies comparing patients with colorectal cancer exhibiting prominent amounts of tumor-infiltrating lymphocytes or not and comparing of survival of CRC patients.
Pts N: patients number; TIL: tumor-infiltrating lymphocytes; IEL: intraepitherial lymphocytes; RFS: relapse-free survival; OS: overall survival; HR: hazard ratio; NR: not reported; NS: not significant

munogenicity may result from a large number of mutated proteins, which can serve as tumor-rejection antigens.

The studies described here suggest that there is a significant host response to CRCs and that the presence of the host response is associated with improved survival. These findings suggest that appropriate immunologic approaches may improve patient prognosis.

VACCINE THERAPY: SPECIFIC IMMUNOTHERAPY FOR CRC

Numerous studies have been done on vaccination in colorectal cancer patients. Among them, representative studies of antigen pulsed dendritic cells (DCs) vaccination (three studies), viral vector based vaccination (one study), personalized peptide vaccination (three studies), and colorectal cancer-specific antigen derived peptide vaccination (two studies) are summarized in Table (**2**).

Peptide-Pulsed Dendritic Cells

Dendritic cells (DCs) are the pivotal antigen-presenting cells (APCs) for triggering T cell immunity. Autologous DCs have been used in cancer vaccines for CRC patients. DC-based vaccines can induce tumor-specific immune responses and objective clinical responses in CRC patients with marginal adverse effects.

Liu *et al.* [11] documented an increased number of CEA-specific T cells in 7 of 10 (70%) CRC patients who received a DC vaccination. Two (20%) of these patients had stable disease for at least 12 weeks, and 1 of these 2 patients experienced a transient decrease in CEA levels during the treatment period. In a study by Weihrauch *et al.*, 17 patients received CEA-derived peptide (CAP-1) or CAP-1-pulsed DCs in combination with chemotherapy (irinotecan/ high-dose 5-fluorouracil (5-FU)/ leucovorin (LV) [12]. Five of these patients experienced a complete response, 1 patient had a partial response, 5 patients had stable disease and 6 patients had progressive disease. Favorable results may depend on concurrent chemotherapy. It is noteworthy that increases in CAP-1-specific T cells were observed in 47% of patients after vaccination, whereas the EBV/CMV recall antigen-specific CD8+ cells decreased by an average of 14% during chemotherapy. In a study by Kavanagh *et al.* [13], 13 patients with advanced CRC were treated with DCs loaded with multiple peptides derived from CEA, MAGE, and HER2/neu. When the T cell responses were examined by enzyme-linked immunospot (ELISPOT) assay, 3 patients had T cell responses to one CEA-derived peptide, and 2 patients had T cell responses to multiple peptides. However, all patients showed progressive disease.

Collectively, these results indicate that DC-based vaccination could be a promising strategy for CRC. However, multiple problems, including high cost, conflicting results, and the large amount of time required for vaccine development, must be addressed before an affordable DC-based vaccination can be developed as a standard treatment. Moreover, reliable biomarkers must be identified, and vaccines and protocols must be standardized.

Viral Vector-Based Vaccine

A recombinant vaccinia virus encoding antigen sequences, such as the CEA gene and gene products, is capable of infecting professional antigen-presenting cells (APCs) and presenting CEA peptides to T lymphocytes in the context of HLA class I and II molecules, which activate the corresponding CD8+ or CD4+ T cells. In a phase I study, the safety of the vaccine was documented, and a CEA-specific T cell response was detected; however, no significant clinical effect was observed [14]. Approaches such as boost vaccination, T cell costimulation, and granulocyte-macrophage colony-stimulating factor (GM-CSF) administration enhanced the CEA-specific T cell responses in the majority of patients [15]. A trend towards an enhanced CEA-specific immune response to vaccination and an increase in progression-free survival and overall survival was documented. However, the subject group consisted of several small cohorts with different types of cancers, including 35 CRCs and 9 lung cancers;

Table 2. Specific Vaccine Trials for Colorectal Cancer

Investigator	Vaccines	Chemotherapy	Pts N	Clinical Response	Survival
Liu [11] (2004)	DC + CEA	-	10	2 SD, 8 PD	NR
Weihrauch [12] (2005)	DC * + CEA	Irinotecan, high-dose 5-FU, LV	17	5 CR, 1 PR, 5 SD, 6 PD	OS 17mo. with survival rate of 35% (6/17)
Kavanagh [13] (2007)	DC + peptides **	-	11	11 PD	NR
Marshall [15] (2005)	Virus expressing CEA + costimulator (TRICOM)	-	35	0 CR, 0 PR	Trend towards enhanced CEA-response and an increase in PFS
Sato [18] (2004)	Peptide (personalized)	-	10	1 PR, 1 SD, 8 PD	NI
Sato [21] (2007)	Peptide (personalized)	TS-1-based	7	1 SD, 6 PD	NI, 2/7 patients still alive at follow-up (17, 30mo.)
Hattori [22] (2009)	Peptide (personalized)	UFT/LV	13	6 SD (3 MR), 7 PD	PFS 10.7 wk (range 5.0-51.0 wk), OS correlated with peptide-specific IgG
Hazama (unpublished data)	Peptides (multiple) ***	FOLFOX	26	13 PR, 12 SD, 1 PD	PFS (has not been calculated)
Okuno (unpublished data)	Peptides	UFT/LV	19	17 SD, 2 PD	PFS (7.2 mo)

*A few patients with DC; ** CEA, MAGE, HER2/neu; *** RNF43, TOMM34, KOC1, VEGFR1, VEGFR2; Pts N, patients number; NR, not reported; NI, not identifiable; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response; OS, overall survival; PFS, progression free survival

therefore, definitive conclusions regarding this method cannot de drawn.

Peptide Vaccines

Rosenberg *et al.* [16] summarized the clinical responses to peptide-based vaccine therapy in 2004. Objective response rates for peptide vaccines and viral vaccines administered to patients with metastatic cancer at the National Cancer Institute (Bethesda, Maryland, USA) were 2.9% (11 of 381 cases) and 1.9% (3 of 160 cases), respectively. In a subsequent study, those trials and other trials of cell-based therapies were analyzed collectively, giving a combined objective response rate of 3.8% (29 of 765 patients, 36 protocols). These results indicate that the classical types of cancer vaccines, including peptide vaccines, do not have a promising future as a new treatment modality for cancer.

Personalized Peptide Vaccines

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific cytotoxic T lymphocyte (CTL) precursors are pre-existent. The initiation of immune boosting through vaccination was better than that of immune priming to induce prompt and strong immunity. Based on this concept, Itoh *et al.* [17] conducted a new regimen that included pre-vaccination measurement of peptide-specific CTL precursors in the circulation, followed by vaccination of only CTL-reactive peptide (CTL precursor-oriented vaccine). In a pilot study, 10 patients with advanced CRC were treated with up to four peptides that had been positive in the pre-vaccination measurement [18]. Post-vaccination peripheral blood mononuclear cells (PBMCs) from 5 patients demonstrated an increased peptide-specific immune response to the peptides. An increased CTL response to cancer cells was detected in post-vaccination PBMCs of 5 patients. Interestingly, antipeptide immunoglobulin G (IgG) became detectable in post-vaccination sera of 7 patients. One patient had a partial response, and another patient had stable disease for 6 months. These results are promising, but the clinical response was not satisfactory. In another protocol, the combination of this type of vaccination with chemotherapy in refractory prostate cancer patients was beneficial. This chemoimmunotherapy may break through the impasse in the clinical efficacy of cancer vaccines [19,20].

In a subsequent study, personalized peptide vaccination in combination with the oral administration of a 5-fluorouracil derivative (TS-1) in advanced CRC/ gastric cancer patients was investigated [21]. Eleven patients who did not respond to prior TS-1-based chemotherapy were enrolled. The combination therapy was generally well tolerated. The vast majority of patients experienced an increase in peptide-specific IgG after the sixth vaccination, irrespective of the dose of TS-1. In the patients who received 80 mg/m^2/day of TS-1, the CTL-mediated cytotoxicity against cancer cells was maintained at the prevaccination level. These results indicate that the standard dose (80 mg/m^2/day) of TS-1 in combination with personalized peptide vaccination does not impede immunological responses in cancer patients and could maintain or augment the immunological responses.

The combination of oral UFT® and UZEL® (LV) is a standard chemotherapy for CRC. UFT is an oral anticancer drug consisting of both Tegafur (FT), a prodrug of 5-FU, and uracil, an inhibitor of degradation of 5-FU. UZEL is an oral