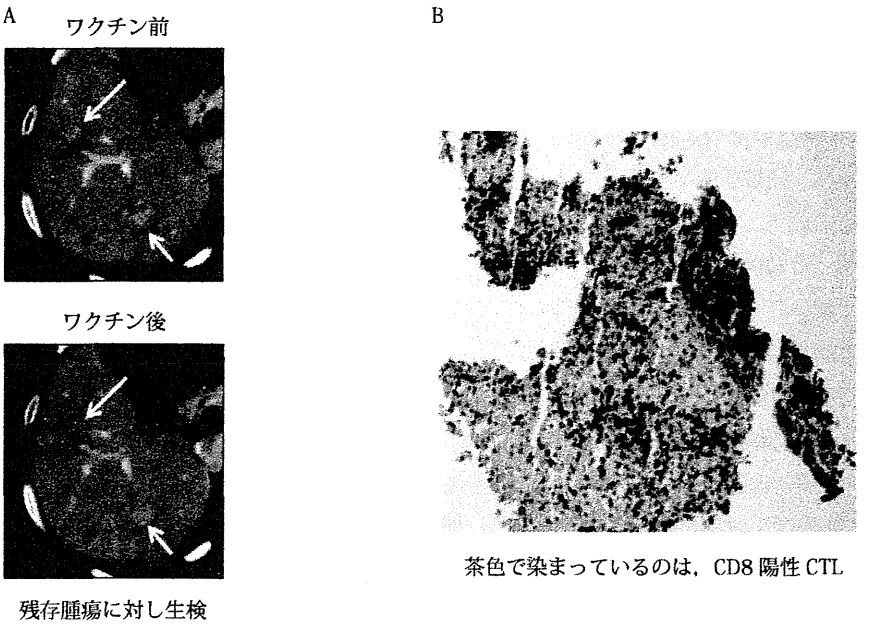
2.3 対象となる患者集団

FDA ガイダンスで「残存病変がない，腫瘍があったとしても少量の患者を対象にしたがんワクチン臨床試験は，ワクチンが抗腫瘍免疫応答をおこすのに十分な時間が確保できる」とあるとおり，進行がんを対象とした場合，病状悪化までの期間が短く抗腫瘍免疫応答が得られない可能性もあり，腫瘍量の少ない患者や術後補助療法が，がんワクチンの適応と考えられてきた。ペプチドワクチン単独での有効性を示すためには，よりがんの量が少ないところを対象にして早期臨床試験を行いたいものである。しかしワクチン療法に限らず，腫瘍量の少ない患者や残存病変のない患者では，多くの費用，症例数と時間がかかってしまうため，早期臨床試験を行うことは困難であるのが現状である。また早期臨床試験で進行がんに対する抗腫瘍効果のデータが乏しい状態で，術後補助療法としての臨床試験を行うことは，倫理面も含めて議論の余地があるところである。

我々は，肝細胞がんの初回手術予定の患者を対象とした術前・術後ワクチン療法の臨床試験を計画している。術前・術後ワクチン投与の臨床試験は，術後ワクチン療法のがん再発抑制効果を評価するほかに，術前の比較的腫瘍量の少ない患者を対象にした際のワクチン療法の奏効率や，手術検体の腫瘍浸潤リンパ球を解析によりワクチン療法の Proof of Concept（POC）を得ることができる。しかし，外科医も未承認薬の術前投与は慎重な姿勢で現在実現していないが，我々は副作用がほとんどないワクチン療法だからこそ可能な最適な臨床試験と主張しており，今後の実現に向けて調整を図っている。

2.4 試験デザイン

日本バイオセラピィ学会ガイダンス（案）「早期探索試験での腫瘍縮小効果が得られない症例でも生命予後延長が得られる可能性があり，早期探索試験をデザインする上で考慮されるべきポイントである」とあり，この内容がもっとも従来の抗腫瘍薬の臨床試験のデザインと異なる，と筆者らは考えている。Long SD（長期間の不変）が CTL による抗腫瘍効果によるものなのか，腫瘍の自然特性をみているだけで意味のないものなのか様々な議論はある。しかし筆者らは，ペプチドワクチン投与後，少ない症例ながらも腫瘍の腫瘍径が一度増大した後縮小をきたした症例や，縮小効果はないものの生検で多数の CD8 陽性 CTL の腫瘍内への浸潤を認めた症例を経験している（図 1）。筆者らの仮説ではペプチドワクチンが抗腫瘍効果を呈する場合 CTL の腫瘍内浸潤は必須であり，このような症例は腫瘍に CTL が浸潤する



A. ワクチン投与前後の CT 画像。肝内腫瘍の一部に消失を認め，残存した腫瘍に対し生検を行った。
B. 生検組織の CD8 免疫染色。残存を認めた腫瘍の中に，多くの CD8 陽性 CTL が，浸潤していた。

図 1　GPC3 ペプチドワクチン臨床第 I 相試験での部分奏功症例　Sawada Y et al, 2012 より改変引用[9]

からこそ観察されるものと考えている。従来の Response Evaluation Criteria in Solid Tumors (RECIST) 評価では，当然このような現象は想定の範囲外である。最近，肝細胞がんでの治療評価に modified RECST が提唱されているが，これは画像上造影効果のある腫瘍部位を viable と評価してその径を評価する方法である[6]。肝細胞がんでは治療後にも腫瘍径として変化はないが，造影効果の変化が生じることが多々あるといった腫瘍特性に沿った評価法である。このように，ペプチドワクチン療法でも，その特性に沿った評価が必要であり，現時点では RECIST は，ペプチドワクチン療法の特性に沿った（たとえば CTL の浸潤までも評価する）画像評価とはいえない。抗腫瘍効果をきたした免疫療法での様々な知見の増加から（腫瘍が大きくなったあと小さくなる，長期間の SD の後に消失など），免疫学的評価基準（Immune-related Response Criteria；irRC）の設定が提唱され，従来の RECIST 評価で PD であっても，免疫学的評価基準の irPR，irSD であれば生命予後は十分期待できる[7]。今後これらの評価方法が確立すると思われるが，現在のところ免疫療法における早期探索試験では，生命予後的指標をエンドポイントとするべきと考えられる。

検証試験はランダム化比較試験で行われ，評価項目は，全生存期間（Overall survival：OS），無病生存期間 DFS（Disease free survival：DFS）にあることは従来の化学療法と変わりないと考える。また化学療法薬などと併用する場合，無増悪生存期間（Progression free survival：PFS）も評価項目になるが，前述のとおりその解釈は困難が予想される。検証試験の方法として，ワクチンの効果がより期待できる腫瘍量の少ない患者を対象に，ファーストラインで抗がん剤や分子標的薬等との併用でワクチンを用い，ワクチン有り無しで OS に差を出すといった方法も考えられる。また現在のワクチン単独療法の奏効率では，適切な患者集団を選択できるバイオマーカーがなければ第Ⅲ相試験の実施は難しいと言われている。しかし，がん種によってはそもそも抗がん剤の奏効率が高いといえないものもあり，そのような抗がん剤との比較試験で，十分 OS に差を出すことは可能でないかと感じている。抗がん剤を使いきって全身状態の悪化した患者にワクチンを使用して OS を伸ばすことを検証するのは難しいかもしれないが，ワクチンの副作用がほとんどないことを勘案すると，これらの患者にこそワクチン療法の意義があるのではないかと考えている。

また HLA 拘束性ペプチドを，当該 HLA を保有する患者のほかに，非当該 HLA を持つ患者に対象群として投与し，HLA を盲検化した HLA-key open 法による試験デザインは，ペプチドワクチンの特性を生かした有用な早期探索試験の方法と考えられている。検証試験としては，HLA のバイアスの可能性，CTL のクロスリアクションによる影響の可能性などが課題として挙げられる。

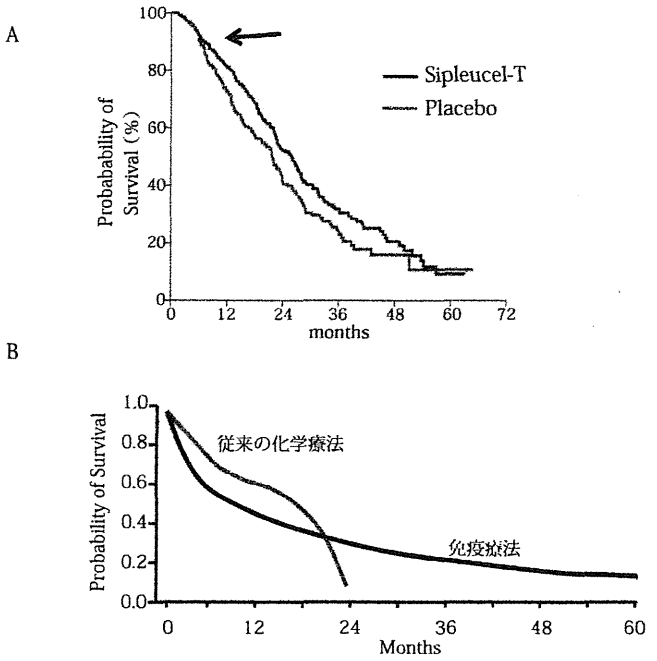
2.5 免疫モニタリング

FDA ガイダンス「早期臨床試験において免疫モニタリングは，提唱された薬理作用の概念実証の確立や投与抗原の免疫原性を示すために行うべきと考える。また抗腫瘍免疫応答のモニタリングに，2 つ以上の免疫モニタリング方法を用いることが推奨される」とあり，免疫モニタリングで得られた結果と臨床効果の関連を示すことが，がん免疫療法の POC になると考えられ，抗腫瘍免疫応答のモニタリングは高いレベルで求められている。

2.6 効果発現の遅延とその解析

FDA ガイダンス「がんワクチンの遅延効果のため，生存曲線は治療後早期には差を示さないが，もしがんワクチンに治療効果がある場合は，後期に曲線の分離が起こることが観察される。統計手法を選択する際，比例ハザード仮説に当てはまらない可能性を考慮する必要がある」とあり，がんワクチン療法では，治療群とコントロール群生存曲線の分離が介入後しばらくした後に観察される（図 2A）。また日本バイオセラピィ学会ガイダンス（案）では，ワクチンの遅延効果によりハザード比が経過中変化する場合，観察期間後期に重みつけを置く，Harmington-Fleming 検定などを用いることを考慮している[5]。一方従来の化学療法薬でも，生存曲線の分離が，後期に現れることがあり，がんワクチンの臨床試験に限って異なる統計解析を行うことは，正しくないという意見もある。しかし後期に重みつけを置くのは，長期生存が見られる免疫療法の特性（図 2B）を含め，解析を行うという意味であり妥当性はあると考える。また筆者らは，生存曲線が後期になって分離する現象は，介入してもまったく変わらない対象かつ早期に死亡する症例（すなわち高度進行がん）が多く存在することに起因すると考えている（通常の化学療法薬の臨床試験でも生じるが，がんワクチンは遅延効果の側面もあり如実に現れる）。現時点のがんワクチンの臨床試験は，高度進行がんには効果をだすのは

難しいかもしれないが，一部の患者に効果があることは間違いがなく，適切な患者集団に行うことができれば，容易に統計学的な有意な差を証明し得ることを示しているともいえるのではないかと考えている。



A. IMPACT studyにおける生存曲線では，プラセボ投与群とがんワクチン投与群の生存曲線の乖離が，矢印の時点で起きている。Kantoff PW et al, 2010より改変引用[1]。
B. 化学療法と免疫療法について模式的に示した生存曲線。従来の化学療法は，奏功後の再発も見られるが，免疫療法では完全奏功，長期生存の可能性を認める。

図2　がんワクチン療法における生存曲線

3.　アカデミアでのがんワクチン開発の経験

我々は，これまで肝細胞がんに対して，GPC3由来ペプチドワクチン療法の医師主導臨床試験を行ってきた[8-14]。基礎研究からのトランスレーショナルリサーチとしての臨床試験という形であったが，幸いに製薬会社への導出という結果につなげることができた我々の経緯を紹介する。

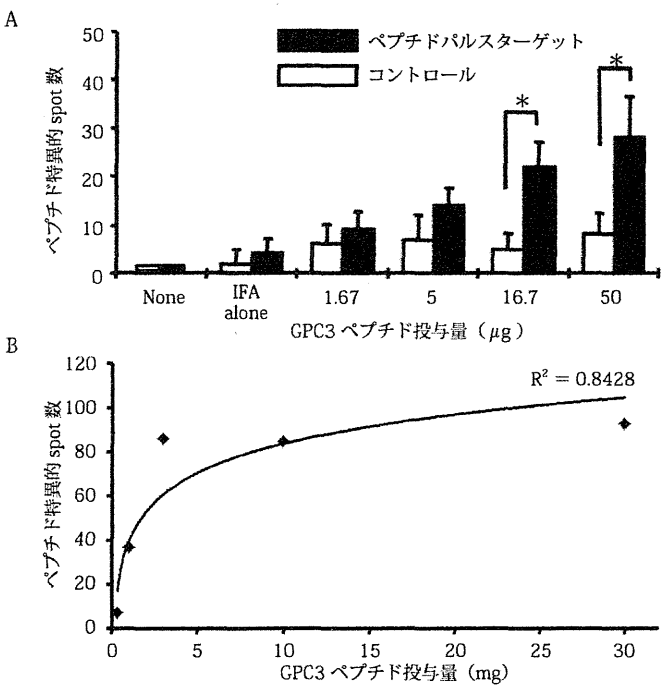
また製薬会社への導出後も，GPC3ペプチドワクチンの医師主導臨床試験は継続しており現在施行中の臨床試験についても紹介する。

3.1　GPC3ペプチドワクチン療法の基礎研究および前臨床試験

我々は，東京大学医科学研究所・ヒトゲノムセンター（中村祐輔教授：現シカゴ大学）との共同研究により，cDNAマイクロアレイのデータを基に，肝細胞がんに特異的な新規がん胎児性抗原としてGPC3を同定した[15]。免疫療法の理想的な標的と考え日本人の約60％が陽性であるHLA-A24拘束性GPC3由来ペプチドを同定し，同様に日本人の約40％が陽性で，欧米人のメジャータイプであるHLA-A2拘束性GPC3由来ペプチドも同定した[16-18]。またマウスを用いた実験において，GPC3抗原の免疫によって，ペプチド特異的CTLが誘導され，抗腫瘍効果は認められたが，自己免疫現象を誘導しないという実験結果を得ることができた[17,18]。

このペプチドを用いた臨床試験を計画するにあたり，従来の前臨床試験にあたるものであるが，ペプチド投与開始量を決定するための最善のプロトコール作成のための実験を行った。当時ペプチド投与量について，免疫応答の強弱はペプチド量には依存しないといった報告はある一方，従来の抗がん剤の試験デザイン同様，臨床試験では3＋3デザインで容量制限毒性の有無を調べるスタイルであった。そこでペプチド投与量によって免疫応答の誘導能に相違が見られ

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



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

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

3.2 GPC3 ペプチドワクチン臨床第 I 相試験[8,9]

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

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

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

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

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

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

無再発率とその 95% 信頼区間

無再発率 $p\ (= n/N)$，$q = 1 - p$

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

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

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

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

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

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

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

おわりに

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

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

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

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Cancer Therapy: Clinical

Clinical Cancer Research

Phase I Trial of a Glypican-3–Derived Peptide Vaccine for Advanced Hepatocellular Carcinoma: Immunologic Evidence and Potential for Improving Overall Survival

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Abstract

Purpose: The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target of anticancer immunotherapy against hepatocellular carcinoma (HCC). In this nonrandomized, open-label, phase I clinical trial, we analyzed the safety and efficacy of GPC3 peptide vaccination in patients with advanced HCC.

Experimental Design: Thirty-three patients with advanced HCC underwent GPC3 peptide vaccination (intradermal injections on days 1, 15, and 29 with dose escalation). The primary endpoint was the safety of GPC3 peptide vaccination. The secondary endpoints were immune response, as measured by IFN-γ ELISPOT assay, and the clinical outcomes tumor response, time to tumor progression, and overall survival (OS).

Results: GPC3 vaccination was well-tolerated. One patient showed a partial response, and 19 patients showed stable disease 2 months after initiation of treatment. Four of the 19 patients with stable disease had tumor necrosis or regression that did not meet the criteria for a partial response. Levels of the tumor markers α-fetoprotein and/or des-γ-carboxy prothrombin temporarily decreased in nine patients. The GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 patients. Furthermore, GPC3-specific CTL frequency after vaccination correlated with OS. OS was significantly longer in patients with high GPC3-specific CTL frequencies ($N = 15$) than in those with low frequencies ($N = 18$; $P = 0.033$).

Conclusions: GPC3-derived peptide vaccination was well-tolerated, and measurable immune responses and antitumor efficacy were noted. This is the first study to show that peptide-specific CTL frequency can be a predictive marker of OS in patients with HCC receiving peptide vaccination. *Clin Cancer Res; 18(13); 3686–96. ©2012 AACR.*

Introduction

While primary liver cancer, which predominantly consists of hepatocellular carcinoma (HCC), is the sixth most common cancer worldwide, it has a very poor prognosis, which makes it the third leading cause of cancer mortality (1). One of the major reasons for the poor prognosis of HCC is the limited availability of treatment options for advanced disease. The molecular-targeted agent sorafenib was recently proven to prolong overall survival (OS) in patients with advanced HCC and has become the standard drug for first-line systemic treatment (2, 3). However, according to Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is quite low, and the incidence of adverse drug reactions is high, especially in elderly patients (4). Moreover, no second-line treatment has been established for patients when sorafenib treatment has failed. Therefore, new treatment modalities are urgently required to prolong survival in patients with advanced HCC while minimizing the risk of adverse reactions.

Immunotherapy is a potentially attractive option for HCC. Many tumor antigens identified in HCC are potential antigens for peptide vaccines (5, 6). However, thus

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

A cancer vaccine that induces CTLs to tumor-associated antigens is a potentially attractive option for hepatocellular carcinoma (HCC). However, thus far, immunotherapy using tumor antigen–derived peptides has not showed a correlation between immunologic responses and antitumor efficacy in clinical trials in patients with advanced HCC. Glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC and correlates with poor prognosis.

In a phase I clinical study, we investigated the safety and antitumor effects of, and immunologic response to, a GPC3-derived peptide vaccine. Our results show that GPC3 peptide–specific CTLs appeared in peripheral blood and that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination.

This is the first study to show that peptide-specific CTL frequency was correlated with overall survival in patients with HCC receiving peptide vaccination. These observations suggest that GPC3-derived peptide vaccines could be a novel therapy for patients with HCC.

far, immunotherapy using tumor antigen–derived peptides has not showed adequate antitumor efficacy in clinical trials in patients with advanced HCC (7–9). The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC (72%–81%) and correlates with a poor prognosis (10–14). We identified HLA-A*24:02–restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) as peptides that can induce GPC3-reactive CTLs without inducing autoimmunity (15, 16). Moreover, by conducting a binding assay, we confirmed that HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can bind to HLA-A*02:06 and HLA-A*02:07. HLA-A24 is the most common HLA class I allele in the Japanese population, and 60% of Japanese individuals (95% of whom have an A*24:02 genotype), 20% of Caucasians, and 12% of Africans are positive for HLA-A24 (17, 18). HLA-A2 is also expressed in Japanese (40%) and other ethnic populations, with an estimated frequency of 50% in Caucasians (17, 19). In a preclinical study using a mouse model, we developed an optimal schedule for human clinical trials of a GPC3-derived peptide vaccine (20). On the basis of these results, we conducted a phase I clinical trial of this GPC3-derived peptide vaccine in patients with advanced HCC. We previously reported that several $GPC3_{144-152}$ peptide-specific CTL clones were established from peripheral blood mononuclear cells (PBMC) of patients vaccinated with HLA-A2–restricted $GPC3_{144-152}$ peptide in this trial (21). We recently completed this phase I clinical trial of the GPC3-derived peptide vaccine. We evaluated the vaccine's safety, tolerability, recommended phase II dose, and immunologic and clinical responses in this trial.

Materials and Methods

Patient eligibility

This phase I trial was approved by the Ethics Committee of the National Cancer Center and was carried out from February, 2007, to November, 2009. Patients with advanced or metastatic HCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of HCC on the basis of imaging modalities or histologic examinations; no expectation of response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–1; age between 20 and 80 years; no prior therapy within 4 weeks; life expectancy $\geq$3 months; HLA-A24- or HLA-A2–positive status, as determined using commercially available genomic DNA typing tests (Mitsubishi Chemical Medience); Child–Pugh liver function class A and B; and adequate organ function (white blood cell count $\geq$3,000/μL, hemoglobin $\geq$8.0 g/dL, platelets $\geq$50,000/μL, total bilirubin $\leq$3.0 mg/dL, aspartate aminotransferase $\leq$200 IU/L, alanine aminotransferase $\leq$200 IU/L, and serum creatinine $\leq$1.5 mg/dL). The following exclusion criteria were applied: massive ascites; known brain metastasis; pregnancy or lactation; known history of HIV infection; clinically serious infection; severe cardiac insufficiency; other active malignancy; history of organ allograft; immunodeficiency or history of splenectomy; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial, based on clinical judgment.

Study design and endpoints

This study was a nonrandomized, open-label, phase I clinical trial with dose escalation of the GPC3 peptides in patients with advanced HCC. HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL; American Peptide Company) was used in HLA-A24–positive patients and HLA-A*02:01–restricted $GPC3_{144-152}$ peptide (FVGEFFTDV; American Peptide Company) in HLA-A2–positive patients. Peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant (IFA; Montanide ISA-51VG, SEPPIC), by intradermal injection on days 1, 15, and 29. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines. Administration of 5 incremental doses of peptide (0.3, 1.0, 3.0, 10, and 30 mg/body) was planned. We planned administer each dose to 6 patients, including at least each 2 patients given HLA-A2 or A24-restricted peptide. The primary endpoint was the safety of peptide vaccination. The secondary endpoints were immunologic responses, clinical outcomes, and determination of the optimal dose of peptide for further clinical trials. This study was approved by the Ethics Committee of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000001395).

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Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
0.3	1	75/M	II	III	0	B	C	TAE, PEI, RFA, S-1	PD	2	9	2402	1	8	+	1+	1+
	2	77/M	IV	IVB	0	A	C	PEI, Proton, TAE, TAI	SD	3	11	2402	0	5	+	1+	1+
	3	67/M	IV	IVB	0	A	–	Ope	SD	3	8	0206/0207	22	20	–	2+	1+
	4	51/M	IIIA	IVA	0	B	B	Ope, TAE, TAI	PD	1	2	0201	0	7	+	NA	NA
	5	62/M	IIIA	III	0	A	–	TAI	PD	2	5	0201	0	9	+	1+	1+
	6	69/M	IV	IVB	0	A	–		PD	0	1	0201	10	9	–	NA	NA
	7	59/M	IIIA	IVA	0	A	B	Ope, TAE, TAI	PD	2	3	2402	0	3	+	1+	1+
	8	55/M	IIIA	III	0	A	C	MCT, PEI, TAE, RT, Sor, S-1	SD	3	17	0201	1	5	+	1+	1+
1.0	9	68/F	IIIC	IVA	0	A	C	PEI, TAE, RFA	SD	4	13	0201	8	8	–	NA	NA
	10	72/M	IIIA	IVA	0	B	C	Ope, MCT, RFA, PEI, Sor, TAE, RT, S-1	SD	4	9	0201	8	51	+	1+	1+
	11	60/M	IIIC	IVA	0	A	C	TAE, RFA	SD	4	9	2402	0	11	+	–	1+
	12	62/M	II	III	0	A	–	RFA, PEI, TAE	PD	2	5	0201/0206	0	12	+	–	1+
	13	44/M	IV	IVB	0	A	B	TAE, RFA, PEI, RT	PD	2	24	2402	6	73	+	1+	2+
	14	42/F	IV	IVB	0	A	–		SD	4	14	2402	1	132	+	2+	1+
3.0	15	67/F	IV	IVB	0	A	–	Ope, PEI, TAE, Proton	SD	5	9	0201	0	23	+	1+	1+
	16	58/M	IIIA	III	0	A	–	Ope, TAE, S-1, TAE	SD	5	7	0201	0	101	+	1+	1+
	17	75/M	IIIC	IVA	0	A	C	RFA, TAE	PD	2	7	2402	0	69	+	–	1+
	18	70/M	IV	IVB	1	A	C	Ope, RT	SD	4	14	2402	0	72	+	1+	1+
	19	76/M	IIIA	III	0	B	C	Ope, TAE, TAI	SD	2	3	2402	31	68	+	1+	1+
	20	73/M	II	II	1	A	–	Ope, TAE	SD	8	>34	2402	0	124	+	1+	1+
10	21	52/M	IV	IVB	0	A	B	Ope, TAE, S-1	SD	4	8	0201	1	100	+	2+	1+
	22	71/M	IIIC	IVA	0	A	–	Ope	SD	4	>32	2402	0	171	+	–	1+
	23	70/M	IV	IVB	0	A	B	Ope, TAI, TAE, PEI	PD	2	6	0201	0	5	+	1+	–
	27	56/M	IV	IVB	0	A	C	TAE, UFT	SD	6	>23	2402	64	69	+	NA	NA
	28	57/M	IIIA	IVA	1	B	C	TAE, RFA, TAI	PD	1	1	2402	0	4	+	NA	NA
	29	68/M	IIIA	IVA	0	A	C	Ope, TAE, TAI	PD	2	4	0201	1	125	+	1+	2+
	33	76/M	IV	IVB	0	A	C	Ope, TAE, MCT, RFA, GEM	SD	4	>16	2402	0	5	+	2+	1+
30	24	75/F	IV	IVB	1	A	C	Ope, RFA, RT	PR	5	12	0207	11	196	+	1+	1+
	25	52/M	IV	IVB	0	A	B	Ope, RFA, TAE, RT, UFT	PD	2	12	0206	2	151	+	2+	2+

(Continued on the following page)

Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response (Cont'd)

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
	26	75/F	II	II	0	B	C	MCT, RFA, TAE, TAI	SD	2	8	2402	0	16	+	NA	NA
	30	69/M	IV	IVB	1	A	—	Ope, TAI, UFT, GEM+CDDP, RT	SD	4	6	2402	2	34	+	1+	–
	31	53/M	IV	IVB	0	B	B	TAE, RFA	SD	4	14	2402	0	7	+	NA	NA
	32	67/M	IV	IVB	0	A	B	Ope, Sor, TAE	PD	2	>17	0201	0	441	+	–	–

Abbreviation: PD, progressive disease; PFS, progression-free survival; PS, performance status.

[a]Stage: staging was carried out according to the TNM classification for HCC (Union for International Cancer Control, UICC) and the Japanese integrated staging system (Liver Cancer Study Group of Japan, LCSGJ).

[b]Hepatic virus infection B. HBsAg was examined by radioimmunoassay. C: HCV was detected by RT-PCR.

[c]Prior therapy: Ope, surgery; TAE, transcatheter arterial embolization; PEI, percutaneous ethanol injection therapy; RFA, radiofrequency ablation; S-1, tegafur, gimeracil, oteracil potassium; proton, proton beam therapy; TAI, transcatheter arterial injection; RT, radiotherapy; Sor, sorafenib; MCT, microwave coagulation therapy; UFT, tegafur plus uracil; GEM, gemcitabine; CDDP, *cis*-diamminedichloroplatinum.

[d]Tumor responses were evaluated according to RECIST guidelines and modified RECIST (mRECIST) assessment. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients.

[e]Number of GPC3-specific CTL spots. The number of GPC3 peptide–specific CTL spots (postvaccination) was the maximum number of spots in an *ex vivo* IFN-γ ELISPOT assay for GPC3 peptide, carried out after vaccination and using 5×10^5 PBMCs.

[f]Expression of GPC3 and HLA class I was determined by immunohistochemistry. Degree of staining of tumor cells for GPC3: –, no reactivity; 1+, weak reactivity; 2+, strong reactivity; NA, not analyzed. Degree of staining of tumor cells for HLA class I: –, no membranous reactivity; 1+, weak membranous reactivity; 2+, strong membranous reactivity; NA, not analyzed.

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Evaluation of toxicity and clinical response

Patients were evaluated for signs of toxicity during and after vaccination. Adverse events were graded according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Hematologic examinations were conducted before each vaccination. The tumor size was evaluated by computed tomography (CT) or MRI before vaccination, and then 1 month after the third vaccination. Tumor responses were evaluated according to the RECIST guidelines and the modified RECIST (mRECIST) assessment (22).

Measurement of immunologic response

Ex vivo *IFN-γ enzyme-linked immunospot assay.* An *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously (21). Briefly, peripheral blood (30 mL) was obtained from each patient before the first vaccination and 2 weeks after each vaccination and centrifuged with a Ficoll-Paque gradient. PBMCs were frozen before immunologic analysis. All PBMCs obtained from an individual patient were incubated in the same plate and analyzed by *ex vivo* IFN-γ ELISPOT assay at the same time. Noncultured PBMCs (5×10^5 per well) were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 hours at 37°C in 5% CO_2. The GPC3 antigen was the HLA-A2–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide or HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL). PBMCs plus HLA-A2–restricted HIV_{19-27} (TLNAWVKVV) peptide (ProImmune) or HLA-A*24:02–restricted $HIV_{583-591}$ (RYLKDQQLL; ProImmune) were used as negative controls. The assays were conducted in duplicate.

Dextramer staining and flow cytometric analysis. The PBMCs were stained with HLA-A*02:01 Dextramer-RPE [$GPC3_{144-152}$ (FVGEFFTDV), HIV_{19-27} (TLNAWVKVV); Immudex] and HLA-A*24:02 Dextramer-RPE [$GPC3_{298-306}$ (EYILSLEEL), $HIV_{583-591}$ (RYLKDQQLL); Immudex] for 10 minutes at room temperature and with anti-CD8-FITC (ProImmune) for 20 minutes at 4°C. Flow cytometry was carried out using a FACSAria cell sorter (BD Biosciences), as described previously (21).

Immunohistochemical analysis. Biopsy specimens were taken from some of the vaccinated patients, each of whom provided informed consent. Specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), HLA class I (clone EMR8/5; dilution 1:2,500; Hokudo), according to the manufacturers' directions.

GPC3 double-determinant (sandwich) ELISA. Double-determinant (sandwich) ELISA of GPC3 was carried out as described previously (10). The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12; BioMosaics Inc.), and anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (#211-GP/CF; R&D Systems).

Statistical analysis

OS rates were analyzed by the Kaplan–Meier method. Prognostic factors were evaluated using the log-rank test and Cox proportional hazard models. All statistical analyses were conducted using the PASW Statistics software, version 18.0 (SPSS Inc.). Statistical significance was defined by a value of P less than 0.05.

Results

Patient characteristics

Thirty-three patients were enrolled in this study (Table 1). None of the patients dropped out because of adverse events caused by peptide vaccination. Two patients (cases 4 and 6) discontinued the regimen after the second vaccination because of liver function impairment resulting from tumor progression. One patient (case 28) could not undergo a CT scan after the third vaccination because of tumor progression. These patients were judged to have disease progression, but were not removed from the analyses at the advice of the effect and safety evaluation committee, including the external members. All patients received adequate follow-up to monitor toxicity. The median follow-up period was 9.0 months (range, 1.1–34.1 months). Of the 33 patients, 28 were male. Their average age was 64.3 years (range, 42–77 years). Five patients had a performance status (PS) of 1; all others had a PS of 0. Staging was conducted according to the tumor-node-metastasis (TNM) classification for HCC (Union for International Cancer Control). Sixteen patients were diagnosed with stage IV disease. Seven patients had Child–Pugh class B disease, and all others Child–Pugh class A disease. Twenty-three patients (70%) had a hepatic virus infection. All but 2 of the 33 patients had undergone conventional chemotherapy, surgery, and transcatheter arterial embolization before receiving GPC3 peptide vaccine therapy. At the time of the trial's initiation, sorafenib had not been approved by the drug administration in Japan. Only a few patients had received sorafenib as prior therapy in this phase I trial. One patient treated with gemcitabine had had stable disease for 5 months immediately before vaccination (case 33). The gemcitabine therapy was discontinued because of nausea and lightheadedness. Other patients had undergone prior therapy, but all of them showed progression of the disease before enrollment in this study.

We evaluated the expression of GPC3 and HLA class I in the primary tumors that could be obtained (Supplementary Fig. S1). GPC3 expression was detected in 21 of 26 patients (81%), consistent with previous reports (10–14). Cell membrane expression of HLA class I was evident in 23 of 26 patients (88%; Table 1).

GPC3 peptide vaccine was well-tolerated

The adverse events observed in this trial are listed in Table 2. Dose-limiting toxicity and dose-specific adverse events were not seen. Grade III hematologic adverse events (impaired liver function) were observed in 4 patients (cases 4, 6, 7, and 23). These 4 patients had progressively massive

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Table 2. The incidence of adverse event

Adverse event	Total (%)	Grade I (%)	Grade II (%)	Grade III (%)
Any event	33 (100)	9 (27.3)	20 (60.6)	4 (12.1)
Any immune-related event	33 (100)	27 (81.8)	6 (18.2)	0
Drug fever	8 (24.2)	4 (12.1)	4 (12.1)	0
Rash or flushing	27 (81.8)	24 (72.7)	3 (9.1)	0
Injection site reaction	33 (100)	33 (100)	0	0
Pruritus	6 (18.2)	6 (18.2)	0	0
Blood	15 (45.4)	6 (18.2)	9 (27.3)	0
Leukopenia	6 (18.2)	2 (6.1)	4 (12.1)	0
Neutropenia	8 (24.2)	5 (15.2)	3 (9.1)	0
Anemia	5 (15.2)	2 (6.1)	3 (9.1)	0
Thrombopenia	3 (9.1)	1 (3.0)	2 (6.1)	0
Increase in PT-INR	2 (6.1)	2 (6.1)	0	0
Hepatic	23 (69.7)	10 (30.3)	9 (27.3)	4 (12.1)
Hyperbilirubinemia	9 (27.3)	3 (9.1)	4 (12.1)	2 (6.1)
Increase in aspartate aminotransferase	14 (42.4)	4 (12.1)	6 (18.2)	4 (12.1)
Increase in alanine aminotransferase	12 (36.4)	10 (30.3)	1 (3.0)	1 (3.0)
Renal	9 (27.3)	6 (18.2)	3 (9.1)	0
Increase in creatinine	4 (12.1)	2 (6.1)	2 (6.1)	0
Proteinuria	6 (18.2)	4 (12.1)	2 (6.1)	0
Other laboratory				
Increase in alkaline phosphatase	9 (27.3)	4 (12.1)	4 (12.1)	1 (3.0)
Hypoalubuminemia	10 (30.3)	7 (21.2)	3 (9.1)	0
Hyponatremia	13 (39.4)	12 (36.4)	1 (3.0)	0
Hyperkalemia	4 (12.1)	4 (12.1)	0	0

Abbreviation: PT-INR, prothrombin time-international normalized ratio.

liver tumors. The effect and safety evaluation committee, including the external members, judged that these events were not related to the treatment, but rather to disease progression. All patients experienced grades I or II local skin reactions at the injection site. Transient immune-related events, including drug fever, rash, and flushing, were observed in most patients. Crotamiton, a scabicidal and antipruritic agent, was prescribed to the 5 patients who had mild itching, but no antipyretic analgesics were prescribed. These results suggest that GPC3 peptide vaccine therapy was well-tolerated.

GPC3 peptide vaccination could induce peptide-specific CTLs in most patients

To determine whether the GPC3 peptide vaccine could induce a specific immune response, PBMCs, obtained from all patients before and after vaccination, were examined by *ex vivo* IFN-γ ELISPOT assay. After the second vaccination, the number of GPC3 peptide–specific CTLs in 5×10^5 PBMCs was increased from 0 to 441 in case 32 (Fig. 1A). As shown in Table 1, we found that the GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 of the 33 patients (91%). GPC3-specific CTL frequency increased in a peptide dose-dependent manner (Fig. 1B). Generally, CTLs for some tumor antigens cannot be directly detected *ex vivo*; they can only be detected after expansion by repeated *in vitro* stimulation with the antigenic peptide on appropriate antigen-presenting cells. This finding can be attributed to the sensitivity of the assay and the low frequency of tumor antigen–specific CTLs (23). Surprisingly, GPC3-specific CTLs were directly detected *ex vivo* without *in vitro* peptide stimulation in almost all patients after GPC3 peptide vaccination.

We also analyzed the GPC3-specific CTL frequency by flow cytometry using the GPC3 peptide, Dextramer. The GPC3-specific CTL frequency is indicated as the percentage of both Dextramer-positive and CD8-positice cells before and after vaccination, as shown in Fig. 1C. After the second vaccination, the frequency of GPC3-specific CTLs increased from 0% to 0.12% in case 32.

In many patients who were vaccinated only 3 times, the GPC3-specific CTL frequency decreased within 2 months after the third vaccination. We could vaccinate 4 or more times in 12 cases. In 9 of these, the GPC3-specific CTL frequency increased after the fourth vaccination (data not shown).

CTLs infiltrated the tumor after GPC3 peptide vaccination

Tumor biopsy was carried out (with informed consent) in 7 patients to evaluate the therapeutic effect after vaccination. We evaluated infiltration of CD8-positive T cells by

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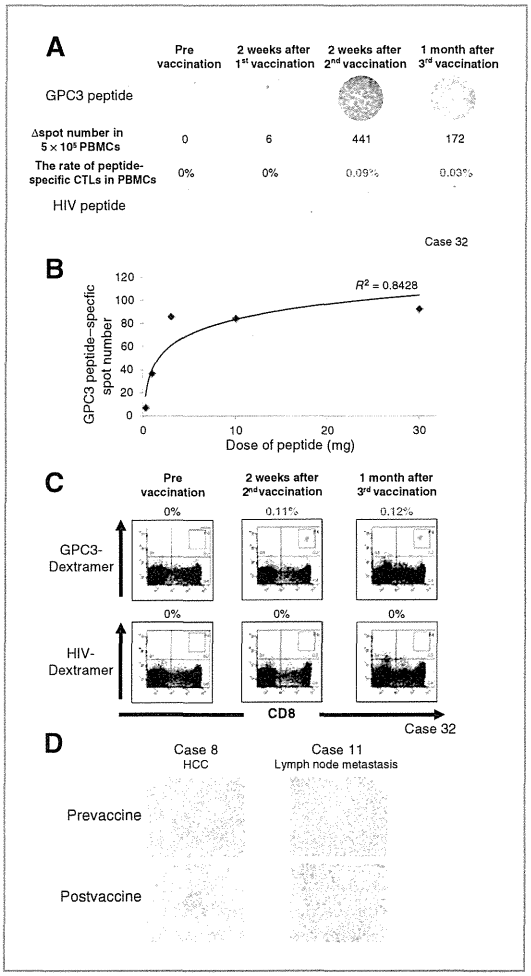

Figure 1. Immunologic monitoring of GPC3 peptide–specific T-cell responses. A, *ex vivo* IFN-γ ELISPOT assay for GPC3 in 5×10^5 PBMCs was carried out before and after vaccination in case 32. The Δspot number indicates the number of GPC3 peptide–specific CTLs. The number of IFN-γ–positive spots increased from 0 to 441 in the wells preincubated with GPC3 peptide. B, median spot number in *ex vivo* IFN-γ ELISPOT assay for GPC3 for each peptide dosage. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. C, *ex vivo* GPC3 Dextramer staining before and after vaccination in case 32. GPC3 peptide–specific CTL frequency is indicated as the percentage of Dextramer-positive CTLs among PBMCs. The frequency of GPC3 peptide–specific CTLs increased from 0% to 0.12% in case 32. D, immunohistochemical staining showing CD8-positive lymphocytes infiltrating tumors before and after vaccination. In cases 8 and 11, CD8-positive T cells (brown) did not infiltrate the tumors before vaccination; in contrast, many CD8-positive T cells infiltrated the tumor after vaccination. Magnification, ×200.

immunohistochemical staining. In case 8, liver biopsy was carried out before and after vaccination. In case 11, neck lymph node metastasis was resected after vaccination. The specimen was compared with an abdominal lymph node metastasis sample obtained by a diagnostic biopsy that this patient underwent before vaccination. While CD8-positive T cells did not infiltrate the tumor before vaccination, marked infiltration of CD8-positive T cells into the tumor was observed after vaccination in both cases (Fig. 1D). In 5 of 7 cases, infiltration of CD8-positive T cells into the tumor was increased after vaccination.

Clinical responses

Patient characteristics and clinical responses in relation to GPC3-specific CTLs are shown in Table 1. Among the 33 patients, one (case 24) was judged to have a partial response (PR) and 19 patients stable disease (SD) for 2 months, according to RECIST. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients. The disease control rate (PR + SD) was 60.6% after 2 months. The median time to tumor progression (TTP) was 3.4 months [95% confidence interval (CI), 2.1–4.6]. The median OS was 9.0 months (95% CI, 8.0–10.0).

In case 24, supraclavicular lymph node metastases markedly regressed, 2 liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (Fig. 2A and B). We carried out a biopsy of the remaining liver tumor and the thoracic bone metastasis after obtaining informed consent. Immunohistochemical staining showed expression of GPC3 and HLA class I on cells in the remaining liver tumor (Fig. 2C). Surprisingly, we detected massive infiltration of CD8-positive T cells into the remaining liver tumor by immunohistochemical staining. No viable tumor cells were found in the biopsy specimens of the thoracic bone metastasis.

Four other patients (cases 1, 15, 16, and 17) had tumor necrosis or partial tumor reduction that did not meet the PR criteria.

Serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are useful tumor markers of HCC (24). The levels of AFP or DCP decreased temporarily at least once in 9 of the 33 patients during the 2-month period (Supplementary Table S1). In 7 of these 9 patients, the levels of DCP fell to less than 30% of baseline values. In 15 of 32 patients, GPC3 protein was detectable in serum before vaccination. The serum levels of GPC3 temporarily decreased at least once in 12 of these 15 patients (data not shown).

These results suggest that there is not the duration of the responses in regards to CTL induction and tumor responses in this phase I trial.

OS was correlated with GPC3-specific CTL frequency

We also examined prognostic factors (Table 3). Fifty GPC3 peptide–specific CTL spots were detected in an *ex vivo* IFN-γ ELISPOT assay conducted using 5×10^5 PBMCs, which means that the GPC3 peptide–specific CTL frequency in peripheral lymphocytes was is 1×10^{-4}%. We focused on these 50 spots to elucidate prognostic factors. Univariate analysis indicated that distant metastasis (−; $P = 0.032$), invasion of the inferior vena cava (IVC) or portal vein (PV;

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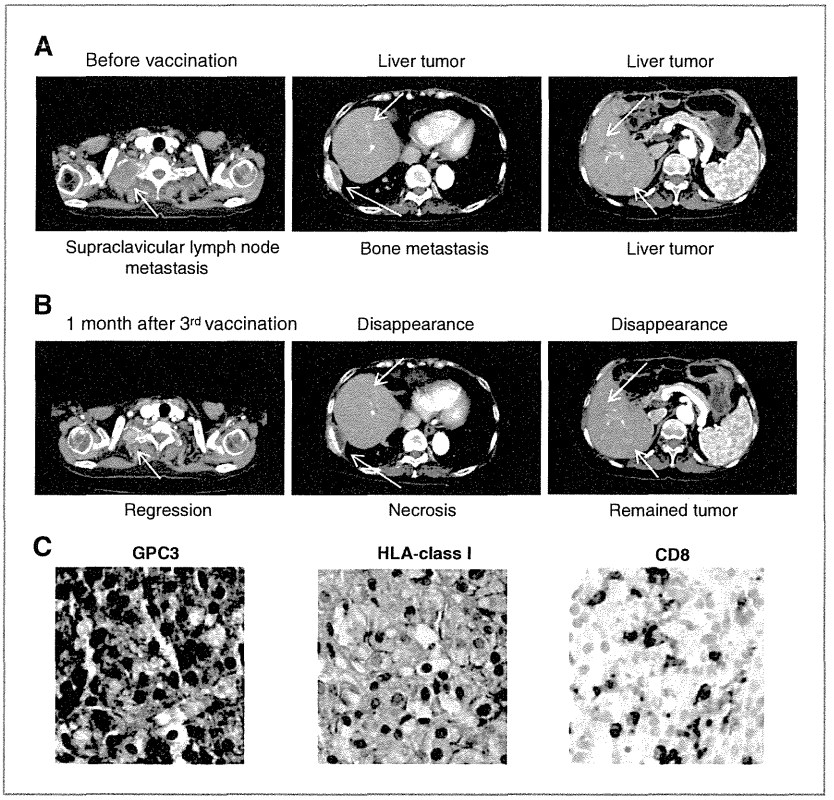

Figure 2. Response assessment in case 24. A, CT imaging, showing liver, pleura, and supraclavicular lymph node metastases before vaccination. B, CT imaging after vaccination was judged as an indicator of a PR. The supraclavicular lymph node metastasis and multiple liver tumors regressed markedly. The pleura metastasis was necrotic. C, we biopsied the remaining liver tumor after vaccination. Immunohistochemical staining showed expression of GPC3 and HLA class I on tumor cells. There was massive infiltration of CD8-positive T cells. Magnification, ×200.

$P = 0.040$), AFP ≥ 100 ng/mL ($P = 0.003$), tumor size ≥ 10 cm ($P = 0.003$), and GPC3-specific CTL frequency < 50 were prognostic factors for OS. Furthermore, AFP ≥ 100 ng/mL ($P = 0.004$; HR = 4.66; 95% CI, 1.61–13.19), tumor size ≥ 10 cm ($P = 0.003$; HR = 4.36; 95% CI, 1.58–12.05), and GPC3-specific CTL frequency < 50 ($P = 0.032$; HR = 2.71; 95% CI, 1.09–6.72) were prognostic factors for OS in a multivariate analysis. We showed that GPC3-specific CTL

Table 3. Prognostic factors of OS

	P univariate	*P* multivariate	HR (95% CI)
Sex (male/female)	0.991		
Age (≥65/<65)	0.608		
Performance status (0/1)	0.707		
Child–Pugh (A/B)	0.063		
Virus infection (+/−)	0.956		
Distant metastasis (+/−)	0.032	0.284	1.71 (0.64–4.54)
Invasion of IVC or PV (+/−)	0.040	0.706	1.21 (0.45–3.30)
AFP (≥100/<100 ng/mL)	0.003	0.004	4.66 (1.61–13.19)
Tumor size[a] (≥10/<10 cm)	0.003	0.005	4.36 (1.58–12.05)
GPC3-specific CTL[b] (≥50/<50)	0.033	0.032	2.71 (1.09–6.72)
HLA (A2/A24)	0.091		
Vaccine[c] (≥1/<1 mg)	0.053		

[a]Tumor size estimated by the RECIST.
[b]The GPC3 peptide–specific CTL frequency examined with *ex vivo* IFN-γ ELISPOT assay in 5×10^5 PBMCs.
[c]The dosage of one vaccine.

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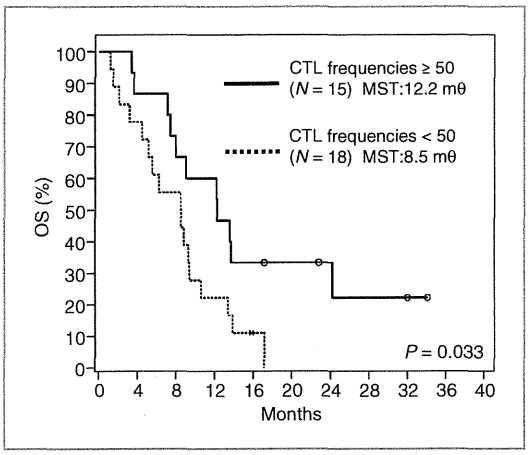

Figure 3. Kaplan–Meier curves for OS. Patients with GPC3-specfic CTL frequencies ≥50 had a longer survival than those with GPC3-specfic CTL frequencies <50 ($P = 0.033$). MST, median survival time.

frequency could be a predictive marker of the effects of GPC3 peptide vaccination. We compared patients with GPC3-specfic CTL frequencies ≥ 50 (N = 15) with those with GPC3-specific CTL frequencies < 50 (N = 18) and found that there was no significant difference in clinical background. We only found a significant difference ($P = 0.004$) for vaccine consumption (≥1.0 vs. <1.0 mg; Supplementary Table S2). Analysis of all 33 patients showed that the median OS was 12.2 months (95% CI, 6.5–18.0) in patients with GPC3-specfic CTL frequencies ≥50, compared with 8.5 months (95% CI, 3.7–13.1) in those with GPC3-specfic CTL frequencies <50 ($P = 0.033$; Fig. 3).

Discussion

We did not observe dose-limiting toxicity in this study. It was difficult to determine the maximum tolerated dose of peptide. A peptide dose of greater than 1.0 mg was required for adequate induction of GPC3-specific CTLs. However, it was complicated to inject more than 10 mg of peptide intradermally because injection mixtures contained both peptide and IFA, and doses of peptide vaccine >10 mg emulsified with IFA (consisting of 2 mL of fluid, including 1 mL of IFA), increased local skin reactions (induration, blushing) at the injection site (Supplementary Fig. S2). Therefore, a dose of peptide of 3.0 mg is recommended for future clinical trials.

We evaluated the expression of GPC3 in the primary tumors of 26 patients by immunohistochemistry. Among the 21 patients with low GPC3 expression (degree of staining − or 1+), one patient was judged to have a PR, and 3 patients have shown long-term survival. We do not suggest that only patients with high GPC3 expression (degree of staining 2+) should be enrolled in further clinical trials.

We studied immunologic responses using an *ex vivo* IFN-γ ELISPOT assay. The GPC3 peptide vaccine induced GPC3-specific CTL responses in 30 of the 33 patients. In contrast, clear immune responses were not observed in patients with HCC in another vaccination trial (9). Differences in tumor antigen may account for the differences in immune response between the 2 vaccination trials. Previous studies have shown that GPC3 is also overexpressed in other malignant tumors, including melanomas, Wilms' tumor, hepatoblastoma, ovarian clear cell carcinoma, and lung squamous cell carcinoma (12, 25–28). GPC3 might also be an effective target for immunotherapy against these tumors (29, 30).

In our study, none of the patients in the 0.1 mg dose group showed more than 50 GPC3 peptide-specific CTL spots. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. Previously, Salgaller and colleagues reported no dose dependency in the capacity of the gp100 peptide to enhance immunogenicity in humans (at doses 1.0–10 mg; ref. 31). In contrast, our data indicate dose dependency in CTL induction, consistent with a previous report using a mouse model (20).

Ten of the 25 patients who received a dose higher than 1.0 mg did not exhibit GPC3-specfic CTL frequencies ≥50. There was no significant difference in the clinical background of patients with GPC3-specific CTL frequencies ≥50 and those with <50. However, GPC3-specific CTL frequency tended to correlate with the serum level of AFP or summed intrahepatic tumor size (Supplementary Table S2). In this study, several patients with advanced HCC exhibited a poor immunologic response to GPC3 peptide vaccination. There are several possible explanations for this poor immunogenicity. HCC is frequently accompanied by cirrhosis, which creates an immunosuppressive environment. There is impairment of the function and maturation of dendritic cells, which has been shown to be related to an imbalance in the extracellular amino acid profile (32). In progressive HCC, the induction of CTL may be suppressed by regulatory T cells or immunosuppressive cytokines (33). It has been reported that GPC3-specific CTLs become exhausted in HCC, and that this exhausted state cannot be reversed by blocking the CTLA-4 and PD-1 inhibitory costimulation pathways (34). Further studies will be necessary to increase the clinical efficacy of immunotherapy for advanced HCC.

The primary endpoint of this study was assessment of the safety of vaccination, but we also showed that tumor antigen-specific CTLs had a crucial role in the immunotherapy against GPC3. GPC3-specific CTL frequency was correlated with OS in this study. Peptide-specific IgG and delayed-type hypersensitivity postvaccination have been reported as potential predictive makers of prolonged survival in patients with advanced cancer vaccinated with peptides (35, 36). However, correlations between immune responses and OS have not been reported in other immunotherapy trials for HCC (7–9, 37). We found that patients with GPC3-specfic CTL frequencies ≥ 50 had a longer survival than those with GPC3-specfic CTL frequencies <50. There was no significant difference in the clinical backgrounds of patients with GPC3-specific CTL frequencies ≥50 and those with <50.

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We clearly showed the presence of GPC3 peptide–specific CTLs in peripheral blood, and showed that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination. The evidence in this study serves as a proof-of-concept for immunotherapy using tumor antigen–specific CTLs. However, we did not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide–specific CTLs. We are currently initiating a pilot study of liver biopsies carried out before and after GPC3 peptide vaccination for advanced HCC to determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide–specific CTLs.

No complete responses were observed when GPC3 peptide vaccination was used as the sole therapy for advanced HCC. To-date, there has been no report of an adequate antitumor efficacy of immunotherapy in clinical trials involving patients with advanced HCC; however, immunotherapy, as an adjuvant after surgical resection, is expected (38). On the basis of this study, we have begun a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC and have also planned combinatorial approaches with chemotherapy.

In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe and indicated a plethora of immunologic responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sawada, S. Mizuno, J. Furuse, T. Nakatsura

Writing, review, and/or revision of the manuscript: Y. Sawada, T. Yoshikawa, D. Nobuoka, T. Takayama, J. Furuse, T. Nakatsura

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Furuse

Study supervision: T. Nakagohri, T. Takayama, K. Yamao, J. Furuse

Monitoring and evaluation of data and safety of the study: K. Uesaka

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A glypican-3-derived peptide vaccine against hepatocellular carcinoma

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The results of a Phase I clinical trial in which a glypican-3 (GPC3)-derived peptide was tested in advanced hepatocellular carcinoma patients point to a strong correlation between immunological and clinical responses. This commentary reviews our fundamental studies and clinical trials on the GPC3-derived peptide vaccine.

The induction of tumor-specific responses in the absence autoimmunity is the ideal goal of immunotherapy. Since the identification of tumor-associated antigens in hepatocellular carcinoma (HCC), immunotherapeutic approaches have been based on the generation of tumor-specific $CD8^+$ T cells that recognize peptides of 8–11 residues derived from intracellular proteins and presented in association with MHC Class I molecules.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans, which are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. We identified GPC3 as a carcinoembryonic antigen and suggested that it would consitute an ideal target for HCC immunotherapy, due to its specific overexpression in HCC (in 81% of paitents) and its correlation with poor prognosis.[1–4] Furthermore, we identified both HLA-A24*(A*2402)*-restricted and H-$2K^d$-restricted $GPC3_{298–306}$ (EYILSLEEL), as well as HLA-A2*(A*0201)*-restricted $GPC3_{144–152}$ (FVGEFFTDV), as peptides that can induce GPC3-reactive cytotoxic T lymphocytes (CTLs) but not autoimmunity.[2,5] HLA-A24 and A2 are the most common MHC Class I alleles in the Japanese population. By performing a binding assay, we confirmed that the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide can also bind to HLA-A*02:06 and HLA-A*02:07. We then conducted a preclinical study in mice to design an optimal schedule for a clinical trial with the GPC3-derived peptide vaccine (**Fig. 1**). This study showed that incomplete Freund's adjuvant (IFA) is indispensable for GPC3 peptide-based immunotherapy, and that the immunological effects of the peptide vaccine are dose-dependent.[6]

Based on these results, we conducted a Phase I clinical trial using this GPC3-derived peptide vaccine in patients with advanced HCC, which has recently been concluded.[7] In this study, 33 patients with advanced HCC received GPC3 peptide vaccination with dose-escalation. Peptides were emulsified with IFA and administered in liquid form by intradermal injection on days 1, 15 and 29. The $GPC3_{298–306}$ peptide was used in HLA-A24-positive patients and the $GPC3_{144–152}$ peptide in HLA-A2-positive patients.

In this trial, we collected evidence of immune responses, demonstrated antitumor effects, and demonstrated the safety of our GPC3-derived peptide vaccine. One patient manifested a partial response (PR) and 4 out of 19 patients with stable disease (SD) exhibit tumor necrosis or regression that did not meet the criteria for PRs. Two months after initiation of treatment, the disease control rate (PR+SD) was 60.6%. When we analyzed the frequency of GPC3-specific CTLs ex vivo by interferon γ (IFNγ) enzyme-linked immunospot (ELISPOT) assays, we could detect GPC3 peptide-specific CTLs in the peripheral blood of most patients. Alongside, we established several $GPC3_{144–152}$ peptide-specific CTL clones from peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial.[8] Tumor biopsies were performed in seven patients to evaluate the infiltration of $CD8^+$ T cells by immunohistochemistry. In five cases, we observed a marked intratumoral infiltration of $CD8^+$ T cells upon vaccination.

A correlation between immunological and clinical responses is nowadays a required as proof for the clinical efficacy of immunotherapy. The frequency of GPC3 peptide-specific CTLs in the peripheral blood correlated with overall survival in HCC patients who received the peptide vaccination. In multivariate analysis, the frequency of GPC3-peptide-specific CTLs constitute the only predictive factor for overall survival in this trial. Analysis of all 33 patients showed a median overall survival of 12.2 mo (95% CI, 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs, compared with 8.5 mo (95% CI, 3.7–13.1) in individuals with a low GPC3-specific CTL frequency ($p = 0.033$). These observations suggest that GPC3-derived peptide vaccines represent a novel immunotherapeutic strategy for patients with HCC, with a potential to improve overall survival.

We subsequently conducted a Phase II study of the GPC3-derived peptide vaccine

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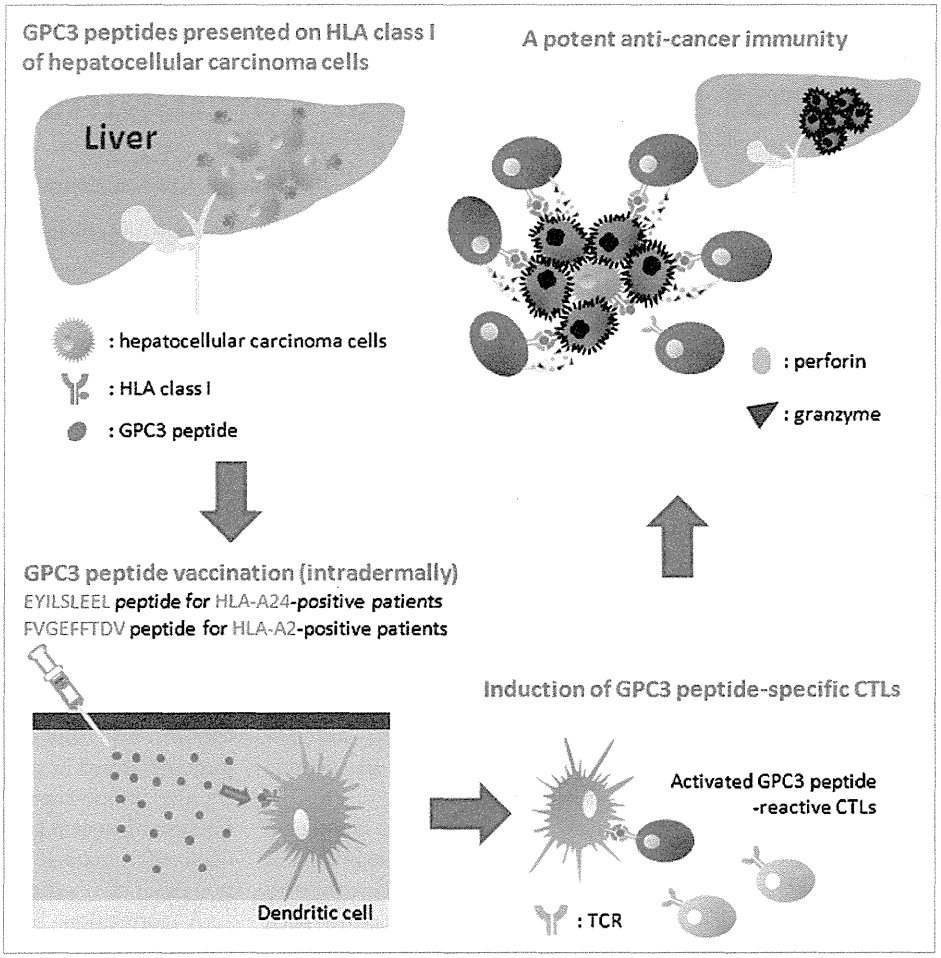

Figure 1. Mechanism of action of the GPC3-derived peptide vaccination. Most patients with hepatocellular carcinoma (HCC) exhibit an HLA-restricted glypican-3 (GPC3)-derived peptide presented in association with MHC Class I molecules. In clinical trials based on GPC3-derived peptide vaccines in HCC patients, the $GPC3_{298-306}$ (EYILSLEEL) peptide was used in HLA-A24-positive patients and the $GPC3_{144-152}$ (FVGEFFTDV) peptide in HLA-A2-positive patients. The peptides were administered with incomplete Freund's adjuvant by intradermal injection, leading to engulfment and cross-presentation by dendritic cells. Dendritic cells are capable of inducing GPC3 peptide-specific cytotocxic T lymphocytes (CTLs), which mediate anticancer immune responses.

as an adjuvant therapy for patients with HCC (UMIN-CTR: 000002614). Forty patients with HCC who had undergone surgery or radiofrequency ablation were enrolled in this Phase II, open-label, single-arm trial. Ten vaccinations were performed over 1 year after curative treatment. Primary endpoints were the 1- and 2-year recurrence rates, while secondary endpoints were immunological responses, as measured by IFNγ ELISPOT. The correlation between the time of recurrence and immunological responses is currently being analyzed.

In the Phase I trial, we did not confirm whether the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific. To address this issue, we are initiating a pilot study of liver biopsies performed before and after GPC3 peptide vaccination for advanced HCC (UMIN-CTR: 000005093).

GPC3 is overexpressed in several malignant tumors, including ovarian clear cell carcinoma (CCC), which is normally characterized by a poor prognosis due to low sensitivity to conventional chemotherapy. We confirmed that a $GPC3_{144-152}$ peptide-specific CTL clone can recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines using an IFNγ ELISPOT assay, and that is can kill ovarian CCC cell lines.[9] We are currently conducting a Phase II study with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

We expect that the results of these trials will provide a rationale for larger randomized clinical trials that determine the efficacy of GPC3-derived peptide vaccines. In addition, as the antitumor effect of the peptide vaccine alone is not dramatic in advanced cancer patients, we aim to develop combinational approaches[9] or strong antigen-specific immunotherapies, including adoptive cell transfer approaches following lymphodepletion.[10] Finally, clinical trials of the adoptive cell transfer of GPC3-specific CTLs in patients with HCC in Japan are planned. Well-designed clinical trials using innovative immunotherapeutic approaches will lead to the development of efficient new therapies for the treatment of GPC3-expressing tumors.