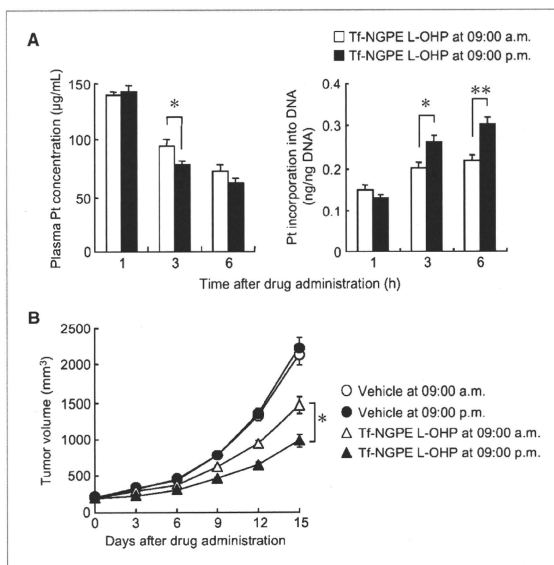


**Figure 6.** Influence of dosing time on the ability of Tf-NGPE L-OHP to inhibit tumor growth in mice. Colon 26 tumor-bearing mice were injected i.v. with a single dose of Tf-NGPE L-OHP (L-OHP: 7.5 mg/kg) or vehicle (9% sucrose) at 9:00 a.m. or 9:00 p.m. **A**, dosing time-dependent differences in the intratumoral delivery of L-OHP by Tf-NGPE liposomes were examined. Plasma Pt concentration (left) and Pt incorporation into tumor DNA (right) were measured at the indicated times after an injection of Tf-NGPE L-OHP. Columns, mean ( $n = 5$ ); bars, SEM; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  for comparison between the two groups. **B**, dosing time-dependent difference in the antitumor effect of Tf-NGPE L-OHP. Points, mean ( $n = 8-10$ ); bars, SEM; \*,  $P < 0.05$  for comparison between the two dosing times.



the cells by receptor-mediated endocytosis. In addition, after a single injection of Tf-NGPE L-OHP, the antitumor effect of the drug varied according to its dosing time. The dosing time dependency of the antitumor effect seemed to be caused by time-dependent changes in the intratumoral delivery of L-OHP by TFR-targeting liposomes.

In the present study, it was shown that the 24-hour rhythm of TFR1 expression in colon cancer cells was controlled by c-MYC, and the cyclical accumulation of TFR1 caused dosing time-dependent changes in the intratumoral delivery of L-OHP by receptor-mediated endocytosis. Identification of the circadian properties of molecules that are targeted by ligand-directed DDS may aid the choice of the most appropriate time of day for their administration.

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## Disclosure of Potential Conflicts of Interest

The authors disclose no conflicts.

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## ファージディスプレイ法を用いた腫瘍組織血管抗体の創製

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## Development of Anti-tumor Blood Vessel Antibodies by Phage Display Method

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Tumor blood vessels are essential for tumor growth. Therefore, these blood vessels are potential targets for anti-cancer therapy. The purpose of this study is to develop anti-tumor endothelial cell (TEC) antibodies for delivering anti-cancer agents or drugs. To achieve this goal, we utilized the phage antibody display library method to create monoclonal antibodies *in vitro*. Accordingly, we developed anti-TEC antibodies from a single chain Fv fragment (scFv) phage display library prepared using the Fv genes amplified from the mRNAs isolated from the TEC-immunized mice. The size of the phage antibody library prepared from the mRNA of the TEC-immunized mice was approximately  $1.3 \times 10^7$  CFU. To select and enrich for the phages displaying the anti-TEC antibodies, cell panning was performed first using the TEC followed by subtractive panning using the normal endothelial cell. After five cycles of panning, the affinity of bound phage clones increased approximately 10 000 folds. Subsequently, clones isolated from the post-panning output library were tested for their antigen-specificity by ELISA and western blotting. One of the scFv phage clones showing antigen-specificity recognized only TEC *in vitro*, and when injected into the Colon26 bearing mice, this clone accumulated more on the tumor tissue than the wild type phage. These results suggest that the isolated an antibody and this clone's target molecule could be potentially useful for novel anti-tumor therapies.

**Key words**—tumor endothelial cell; phage display; antibody

## 1. はじめに

医療技術の進歩に伴い、各種疾患の治療成績は年々向上している一方で、「悪性新生物：がん」は1981年にわが国における死因の第1位となつて以来、その死亡者は年々増加し、現在では死因全体の約3割を占めている。このように、「がん」を克服

できていない現状から、新たながん治療法、診断法の確立が待望されている。

腫瘍組織は、その体積が1 mm<sup>3</sup>を超えて増大する際、血管より最も離れた中心部位は低酸素状態にある。<sup>1-3)</sup>そして、HIF等を介してその低酸素シグナルは腫瘍細胞に伝わり、腫瘍細胞がVEGF、b-FGF等の血管新生誘導因子を産生し、新たな血管を既存の血管より誘導してくることが知られている。<sup>4-7)</sup>この腫瘍により誘導された血管は、腫瘍細胞への酸素・栄養の供給、腫瘍細胞からの老廃物の除去という腫瘍にとってまさにライフラインとも言うべき機能をしており、腫瘍組織の維持、増大に必要不可欠である。<sup>8,9)</sup>さらに、この腫瘍組織血管の誘導は腫瘍の増大だけでなく、腫瘍血行性転移の経路

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本誌説は、日本薬学会第129年会シンポジウムGS6で発表したものを中心に記述したものである。

としても機能している。このように腫瘍組織血管は腫瘍組織にとって必要不可欠な存在であるため、腫瘍組織血管に対して傷害を誘導することは腫瘍組織に致死的なダメージを与えることになり、腫瘍の退縮及び、転移の抑制が期待できる。このような背景も相まって、現在、この腫瘍組織血管を標的とするような分子標的医薬品の開発が盛んに進められている。

近年、疾患関連分子に対する分子標的治療を可能とするモノクローナル抗体が脚光を浴びている。その優れた抗原特異性から、モノクローナル抗体は診断薬<sup>10-14)</sup>として、さらには最近、抗体医薬として応用が非常に注目され、研究、臨床開発が盛んに行われており、現在、基礎研究段階のものを含めれば、約400以上もの抗体医薬品の研究開発が進められているとされている。<sup>15-20)</sup> また、2007年の抗体医薬品の世界市場は約263億3800万ドル(約2兆3800億円)で、2013年には約490億5500万ドル(約4兆4300億円)まで拡大すると予想され、まさに熾烈な研究合戦が世界規模で展開中である。本邦においては、乳がんにおいて過剰発現が認められるerbB2を標的とするTrastuzumab (Herceptin)<sup>9)</sup>、<sup>21-23)</sup> がんの血管新生をターゲットとした抗VEGF中和抗体であるBevacizumab (Avastin)<sup>9)</sup>、<sup>24,25)</sup>を始め、徐々に抗体医薬品が承認・上市されつつある。

腫瘍組織血管は正常組織血管と異なった性質を有している<sup>26,27)</sup>ため、その細胞膜表面若しくは、分泌タンパク質に特異的なタンパク質、いわゆるバイオマーカーが存在している可能性が考えられるが、腫瘍組織血管特異的なバイオマーカー、及びその抗体については世界的に認められる分子はいまだに発見されていないというのが現状である。腫瘍組織血管特異的ではないが、唯一の重要な指標となっているのは血管新生において非常に重要なシグナル系であるVEGFファミリーのVEGFR1 (Flt-1)、VEGFR2 (Flk-1/KDR)の発現上昇のみである。<sup>28-30)</sup> そこで本研究では、新規がん治療薬、腫瘍組織血管特異バイオマーカー探索の強力なツールとなり得る、腫瘍組織血管特異タンパク質に対する抗体の創製を従来のハイブリドーマ法と比較して画期的な抗体創製法であるファージディスプレイ法を用いて試みた。

## 2. ファージディスプレイ法

ファージディスプレイ法は1985年にSmithらに

よって報告され、<sup>31)</sup> バクテリオファージ内に存在するファージミッドベクターへ外来遺伝子(ペプチド・タンパク質・抗体等)を導入することで、バクテリオファージ表面に目的分子を発現させることを可能とする技術であり、その分子はバクテリオファージのコートタンパク質(g3p)と融合した形態で発現している。抗体作製への応用は1990年にMcCaffertyらが抗体機能ドメインを提示したファージディスプレイ法をハイブリドーマ法に代わる新しいモノクローナル抗体作製技術として報告したことに始まる。<sup>32)</sup> 1991年にはMarksらが免疫していないヒトの末梢血リンパ球を出発材料に構築したファージ抗体ライブラリから、ヒト生体成分や、ターキー卵白アルブミン、ウシ血清アルブミンなど異種動物の抗原に対するファージ抗体の分離を報告した。<sup>33)</sup>

繊維状ファージM13は環状の一本鎖φノムDNAを持ち、そのまわりに5つのコートタンパク質(g3p, g6p, g7p, g8p, g9p)が連結した細長い筒状の構造をしており、大腸菌に感染して増殖するウイルスである。ファージディスプレイは、これらのファージコートタンパクと外来ポリペプチドを融合した形で発現させることでファージ表面にディスプレイさせる方法である。

ファージディスプレイ法の特徴は、①任意の外来分子をファージ表面に提示できること、②1個の宿主菌に1個のファージしか感染しないため(pDNA incompatibilityに寄与する)、各ファージ内の外来遺伝子とファージ表面に提示された外来遺伝子産物が一致していること、③種々の外来遺伝子産物を提示したファージを数億種類以上の多様性を有するライブラリとして容易に、かつ短期間(1週間以内)で調製できること、④宿主菌に感染させることで簡便に特定のファージを増幅できることにある。<sup>34,35)</sup>

外来遺伝子として一本鎖抗体(single chain variable fragment: scFv)遺伝子を用いるファージ抗体ライブラリは、ファージ表面に数十万から数億以上のレパートリーを有する抗体機能性ドメインであるVL領域とVH領域をリンカーで連結したscFv抗体を発現させるものである(図.1)。このライブラリから標的タンパク質へ特異的に結合するクローンを選択・回収し、増幅する操作(パンニング)を繰り返すことによって、標的タンパク質に結合する

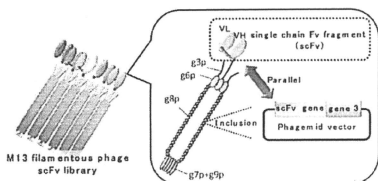


Fig. 1. The scFv Phage Display System

scFv 抗体分子を表面提示したファージをスクリーニングすることが可能である。<sup>36,37)</sup> しかも得られたファージは目的抗体をコードする遺伝子を内封しているため、抗体の遺伝子配列も同時に獲得することが可能である。このように、ファージディスプレイ抗体ライブラリは *in vitro* で生体内の抗体産生系を模倣し、さらに生体の免疫系から独立した抗原の種類を選ばない優れた抗体創製システムとして、抗体医薬開発に大きく貢献している。

### 3. 抗腫瘍組織血管抗体の創製

**3-1. 腫瘍組織血管内皮細胞モデル** 抗腫瘍組織血管抗体を創製するためには、腫瘍組織血管内皮細胞を獲得しなければならないが、腫瘍組織血管内皮細胞のみを生体内腫瘍組織から単離することは非常に困難である。そこで筆者らはがん細胞の培養上清 (Conditioned Medium: CM) を用いることで、*in vivo* の腫瘍組織血管内皮細胞を模倣した腫瘍組織血管内皮細胞モデルを *in vitro* の系で再構築し、このモデル細胞から抗原タンパク質を調製後、これを用いて抗体創製を試みた。本検討ではマウス結腸がん細胞 (Colon26) の CM で培養したヒト臍静脈血管内皮細胞 (HUVEC) を腫瘍組織血管内皮細胞モデル (Colon26 CM-HUVEC) とした (Fig. 2)。

この腫瘍組織血管内皮細胞モデルは、生体内腫瘍組織血管内皮細胞に特徴的な性質である細胞間透過性の亢進を示し、<sup>38)</sup> また腫瘍組織血管内皮細胞モデルの膜抗原を樹状細胞にパルス後、この樹状細胞をマウスに免疫したところ、*in vivo* において、副作用を引き起こさなく腫瘍組織血管特異的な傷害性が誘導され、それに伴う抗腫瘍効果が観察された (未発表データ)。さらには、2 次元電気泳動解析により、その特異的抗原タンパク質の存在が明らかとなった (未発表データ)。

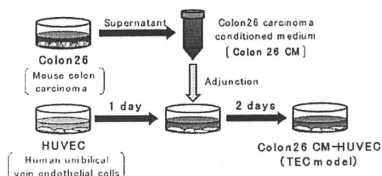


Fig. 2. Development of the Tumor Endothelial Cell Model  
Human umbilical vein endothelial cells (HUVEC) cultured in Colon26 carcinoma-conditioned medium (Colon26 CM-HUVEC) were used as model TECs.

これらのことから、この腫瘍組織血管内皮細胞モデルは生体内の腫瘍組織血管内皮細胞と全く同じ特異的抗原タンパク質を発現している可能性が非常に高いと考えられる。

**3-2. 腫瘍組織血管内皮細胞モデル免疫抗体ライブラリの作製** 現在、ファージ抗体ライブラリとしては、免疫していない動物や健康人の B 細胞を由来とした「ナイーブファージ抗体ライブラリ」<sup>39,40)</sup> と免疫した動物や疾患患者の B 細胞を由来とした「免疫ファージ抗体ライブラリ」<sup>41)</sup> の 2 種類が主に用いられている。各ライブラリにはそれぞれ長所・短所があり、「ナイーブファージ抗体ライブラリ」は抗体遺伝子に偏りのない多様性に富んだ抗体ライブラリを獲得できるという長所を持つ一方で、抗原結合力の強い抗体を得ることが困難であるという短所も持ち合わせている。また、「免疫ファージ抗体ライブラリ」は抗原結合力の強い抗体を得ることができるという長所を持つ一方で、免疫状態に偏りがあるため、「ナイーブファージ抗体ライブラリ」と比較して得られる抗体ライブラリは多様性に乏しいという短所を持つ。このように両ライブラリは一長一短であるため、目的に合わせて使い分ける必要がある。

本検討では最終的に獲得した抗体を抗原探索・解析のツールとしてだけでなく、ドラッグデリバリーのツールとして用いる狙いがあるため、より強力な結合力を有する抗体を獲得可能な「免疫ファージ抗体ライブラリ」を作製した。

免疫原として腫瘍血管内皮細胞モデルである Colon 26 CM-HUVEC を BALB/c マウスに一週間おきに 2 回免疫を行った。次に、マウスの Colon26 CM-HUVEC に対する抗体価を測定後、脾臓を回収

し、mRNAを回収した。続いて、このmRNAをテンプレートにcDNAを作製し、PCRにより抗体のVL、VH領域のDNAの増幅を行った。さらに、増幅したVL、VH領域のDNAを連結させるassembly PCRを行い、scFv DNAを作製した。そして、このscFv DNAをファージミドベクター (pCANTAB5E) ヘクローニングした。最後にクローニング後のファージミドベクターを大腸菌 (TG1) ヘレクトロポレーションにより導入した。構築したライブラリのライブラリサイズは大腸菌の形質転換効率より算出した。その結果、構築した抗体ライブラリは  $1.3 \times 10^7$  CFU という抗体多様性を保持していた (Fig. 3)。

### 3-3. 抗腫瘍組織血管抗体のスクリーニング

機能性分子を獲得するためのスクリーニング系の設計はファージディスプレイ法のみならず、ディスプレイ技術を駆使する場合のキーテクノロジーとして重要視される。精製抗原が入手可能な場合は、固層化法 (パンニング) やビオチン化抗原とストレプトアビジン固定化担体を用いたスクリーニング方法が主要な選択系として用いられている。しかし、各スクリーニングを効率よく行うためには、対照抗原の目的エピトープを被覆、あるいは変性させない工夫が必要であり、さらには反応温度や洗浄条件、塩濃度など複数の要因が影響する。

今回、われわれは精製された抗原タンパク質を獲得しているため、抗体ライブラリから抗腫瘍組織血管抗体を選別するスクリーニング系として簡便なバ

ンニングを選択した。パンニングはファージ抗体ライブラリ内から、固層化した標的タンパク質へ特異的に結合するクローンを選択・回収し、増幅する操作であり、このパンニングを繰り返すことによって、標的タンパク質に結合するscFv抗体分子を表面提示したファージを選別・濃縮することが可能である (Fig. 4)。

近年、臨床で用いられている抗体医薬品は細胞膜表面の膜タンパク質、若しくは血中に遊離している機能性タンパク質 (レセプターアゴニスト等) を認識する抗体である。今回創製する抗体の将来的な展望として、創出した抗体をドラッグデリバリー、また診断のツールとして使用することを目的としているため、創製する抗体は細胞内タンパク質ではなく、細胞外表面上の膜タンパク質を認識する抗体であることが必須条件であると考えられる。このことから、われわれはパンニングにより抗体を選別する際に用いる標的抗原には、調製時に破壊された細胞の細胞内タンパク質が混入する恐れのある、細胞抽出抗原タンパク質 (細胞ライゼート、ブタノール抽出膜抗原タンパク質等) を用いるのではなく、生細胞をそのまま標的抗原として用いることが最適であると考えた。

さらに、抗腫瘍組織血管抗体を創製するにあたって、われわれが非常に重要であると考えていることは、いかに副作用の少ない抗体を選別するかである。腫瘍組織血管は正常組織血管内皮細胞が腫瘍細胞の産出する液性因子 (サイトカイン、ケモカイン

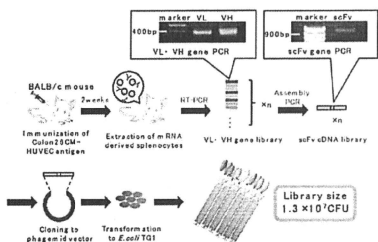


Fig. 3. Development of the Tumor Endothelial Cell Immune Antibody Library

The single chain antibody fragment variable (scFv) phage display library was prepared by amplifying the Fv genes from the mRNA derived from the TEC-immunized murine splenocytes.

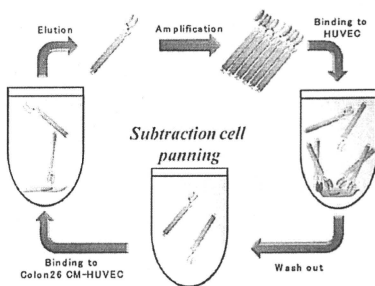


Fig. 4. Antibody Selection by Subtraction Cell Panning  
Anti-TEC antibodies were selected by cell panning against Colon26 CM-HUVEC with subtractive panning against normal HUVEC.

ン、増殖因子等)により腫瘍組織へ誘導され、増殖することで、形成されると考えられている。このため、腫瘍組織血管内皮細胞は正常組織血管内皮細胞と構成タンパク質の大部分が類似していると考えられる。事実、われわれが構築した腫瘍組織血管内皮細胞モデルにおいても、2D-DIGE 比較解析の結果、正常組織血管内皮細胞と構成タンパク質の大部分が類似していた。このことから、パンニングの際、腫瘍組織血管内皮細胞モデルのみを用いてしまうと、正常組織血管内皮細胞と交差性を持つ抗体が選別されてくる可能性が非常に高い。そこで、腫瘍組織血管内皮細胞モデルと抗体ライブラリファージを結合させる前に、正常組織血管内皮細胞と結合させるサブトラクションセルパンニングを行うことで腫瘍組織血管内皮細胞モデル特異抗体の選別・濃縮を図った (Fig. 4)。

まず、抗体ライブラリファージを HUVEC と結合させ、正常組織血管と結合する抗体ファージを除去し、遊離している抗体ファージを Colon26 CM-HUVEC と結合させた。非特異的結合ファージを洗浄後、Colon26 CM-HUVEC 結合抗体ファージを回収し、大腸菌に感染させ、増幅した。このサブトラクションパンニングを計 5 回行った結果、パンニング前のファージ抗体ライブラリ (input ファージ) とパンニングにより回収されたファージ (output ファージ) の比率は、5th パンニング時では、1st パンニング時と比較して、約 10000 倍という高い比率を示したことから、Colon26 CM-HUVEC 特異的に結合する抗体ファージの選別・濃縮に成功したと推測される (Fig. 5)。

さらに、抗体アミノ酸配列を確認するため、各パンニングラウンドのファージ抗体をモノクローン化後、インサート PCR を行い、scFv DNA の増幅が確認されたクローンの scFv アミノ酸シーケンスを解析した。その結果、2 種類の scFv のアミノ酸シーケンス解析に成功した (未発表データ)。獲得した 2 種類の scFv は抗体の抗原認識性を決定する上で非常に重要である 3 か所の相補性決定領域 (complementarity-determining regions: CDR1, CDR2, CDR3) を保有し、最も多様性に富むとされている CDR3 領域の中で特に、VH の CDR3 領域においては、アミノ酸の種類、数ともに全く異なっていた。

#### 3-4. 獲得抗体の特性評価 獲得抗体の *in vitro*

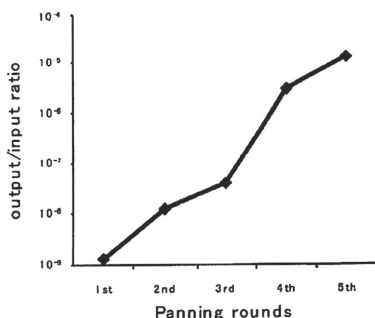


Fig. 5. Enrichment of Antibodies to Tumor Endothelial Cell Model by Subtraction Cell Panning

における抗体特性評価として、ELISA, western blotting による抗原結合性評価、及び抗原分子量の探索を行った (Fig. 6)。

ELISA による抗原結合性評価の結果、Clone 1 ファージ抗体は、Colon26 へは結合性は示さない一方で、Colon26 CM-HUVEC へは HUVEC への結合性と比較して約 2 倍の抗原結合性を示した。さらにこのファージ抗体の抗原分子量を探索するため、Clone 1 ファージ抗体を用いて western blotting を行った。

Western blotting の結果、Clone 1 ファージ抗体は分子量約 45000 Da のタンパク質に対して結合性を示し、そのバンドの発光強度は ELISA での結果とほぼ相関していた。また、この抗原タンパク質は腫瘍組織血管に高発現しているとされている VEGFR2 とは異なる分子量を示していたことから、VEGFR2 以外の新規腫瘍組織血管マーカーである可能性がある。

さらに獲得抗体の *in vivo* での腫瘍組織集積性を評価するため、獲得ファージ抗体を担がんマウスへ静注し、全身灌流後、腫瘍組織を抽出した。その腫瘍組織内のファージ titer を計測することで獲得抗体の腫瘍組織集積能を評価した。*In vitro* における Colon26 CM-HUVEC への抗原結合性を示した Clone 1 ファージ抗体を担がんマウスへ投与したところ、有意差は得られなかった ( $p=0.08$ ) が Clone 1 ファージ抗体は野生型ファージと比べて約

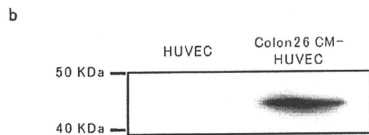
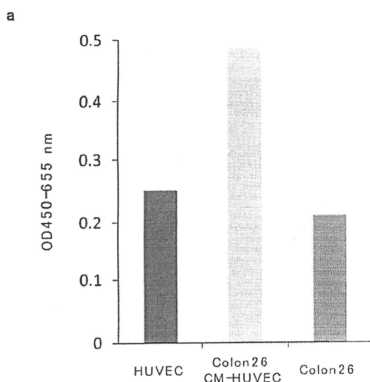


Fig. 6. Evaluation of Antibody Binding Activity *In Vitro* (a: ELISA, b: Western blot). The binding specificities of the phage antibodies were assessed *in vitro* by using ELISA and Western blotting.

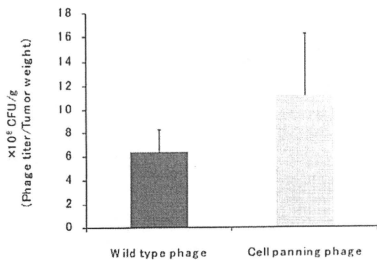


Fig. 7. Evaluation of Antibody Binding Activity in a Pilot Study *In Vivo*

The binding specificities of the phage antibodies were assessed *in vivo* by analyzing their accumulation on the tumor tissue.  $n=3$ ,  $p=0.08$ .

1.8 倍の腫瘍集積性を示す傾向にあった (Fig. 7)。この有意差が得られなかった原因は、抗体をファーゼ上に提示した状態で用いていることから、ファーゼが持つ非特異的吸着性の影響を受け、本来得られ

るべき差異がマスクされてしまったためとわれわれは推測している。そこで今後、より詳細に獲得した抗体の抗原認識性を評価するため、得られた Clone 1 の遺伝子をもとに、抗体のみを精製し、再検討する予定である。なお、Clone 2 についても現在、同様に特性評価を行っている。

#### 4. おわりに

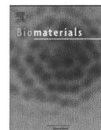
本研究の結果、抗腫瘍組織血管抗体の創製に成功した。今後は免疫沈降法、質量分析法を駆使して各抗体の抗原タンパク質を同定し、それら抗原タンパク質の機能、生体分布評価を行う予定である。将来的に本研究により創製した抗体、その抗原が、がん治療、診断及び腫瘍組織血管研究の進展に大きく貢献することを期待している。

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## Development of an antibody proteomics system using a phage antibody library for efficient screening of biomarker proteins

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### ABSTRACT

Proteomics-based analysis is currently the most promising approach for identifying biomarker proteins for use in drug development. However, many candidate biomarker proteins that are over- or under-expressed in diseased tissues are found by such a procedure. Thus, establishment of an efficient method for screening and validating the more valuable targets is urgently required. Here, we describe the development of an "antibody proteomics system" that facilitates the screening of biomarker proteins from many candidates by rapid preparation of cross-reacting antibodies using phage antibody library technology. Using two-dimensional differential in-gel electrophoresis analysis, 16 over-expressed proteins from breast cancer cells were identified. Specifically, proteins were recovered from the gel pieces and a portion of each sample was used for mass spectrometry analysis. The remainder was immobilized onto a nitrocellulose membrane for antibody-expressing phage enrichment and selection. Using this procedure, antibody-expressing phages against each protein were successfully isolated within two weeks. The expression profiles of the identified proteins were then acquired by immunostaining of breast tumor tissue microarrays with the antibody-expressing phages. Using this approach, expression of Eph receptor A10, TRAIL-R2 and Cytokeratin 8 in breast tumor tissues were successfully validated.

These results demonstrate the antibody proteomics system is an efficient method for screening tumor-related biomarker proteins.

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### 1. Introduction

Proteomics-based analysis is the most promising approach for identifying tumor-related biomarker proteins used in the drug development process [1–3]. The technological development of proteomics to seek and identify differentially expressed proteins in disease samples is expanding rapidly. However, in spite of the identification of many candidate biomarkers, the number of biomarker proteins successfully applied to drug development has been limited. The main difficulty is the lack of a methodology to comprehensively analyze the expression or function of many candidate proteins and to efficiently select potential biomarker

proteins of interest. To circumvent this problem, an improved technology to efficiently screen the truly valuable proteins from a large number of candidates is desirable.

Monoclonal antibodies are extremely useful tools for the functional and distributional analysis of proteins [4–6]. For example, they can be applied to the specific detection and study of proteins through various techniques including ELISA, Western blotting, fluorescent imaging and tissue microarray analysis (TMA). Of all these techniques, TMA is particularly valuable because it enables the analysis of clinical expression profiles of antigens from many clinical samples [7–11]. However, the common hybridoma-based antibody production is a laborious and time-consuming method. Thus, it is impractical to create antibodies against many differentially expressed proteins identified by proteomics technologies, such as two-dimensional differential in-gel electrophoresis (2D-DIGE) [12–15]. Furthermore, a relatively large amount of antigen (several milligrams) is necessary to produce an antibody (i.e., immunization of animals or screening of positive clones). The

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production of protein on this scale often requires engineering the corresponding gene for heterologous expression, which may require some time to optimize. In this respect, phage antibody library technology is able to construct a large repertoire protein or peptide consisting of hundreds of millions of molecules. Monoclonal antibodies against target antigens are then rapidly obtained from the phage libraries displaying single chain fragment variable (scFv) antibodies *in vitro* [16–21].

However, the amount of protein in spots detected by 2D-DIGE analysis is generally very small (hundreds of nanograms). Therefore, a technology for generating monoclonal antibodies from such small amounts of antigen needs to be developed. There are no reports that describe the successful isolation of antibodies against small amounts of proteins obtained from differential proteome analysis.

Here, we report the establishment of a method for the efficient isolation of scFv antibody-expressing phages from a small amount of protein antigen prepared via 2D-DIGE spots using a high quality non-immune mouse scFv phage library [22]. We also describe an efficient method for screening and validating tumor-related biomarker proteins of interest from a number of differentially expressed proteins by expression profiling using TMA and scFv antibody-expressing phages.

## 2. Materials and methods

### 2.1. Non-immune mouse scFv phage library

Construction of the improved non-immune murine scFv phage library has been described previously [22]. The phage library was prepared from a TG1 glycerol stock containing the scFv gene library.

### 2.2. Affinity panning using BiACore® and nitrocellulose membrane

Three different amounts (5000 ng, 50 ng or 0.5 ng) of KDR-Fc chimera (R&D systems Inc., Minneapolis, MN) or a portion of the proteins (1–5 ng) extracted from 2D-DIGE spots were immobilized on a BiACore sensor chip CM3® (BiACore, Uppsala, Sweden) or on a nitrocellulose membrane. BiACore-based panning has been described previously [22]. Membrane-based panning was performed using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with blocking solution (10% skimmed milk, 25% glycerol) for 2 h and then washed twice with 0.1% TBST (Tris-buffered saline containing 0.1% Tween 20). The model phage library (anti-KDR scFv antibody-expressing phages: wild type phage = 1:100) or the non-immune scFv phage library was pre-incubated with 90% blocking solution at 4 °C for 1 h and then applied to each well. After 2–3 h incubation, the apparatus was washed ten times with TBST. Bound scFv antibody-expressing phages were then eluted with 100 mM triethylamine. The eluted phages were incubated in log phase *E. coli* TG1 cells and glycerol-stocks prepared for further repeat panning cycles. Phage titer was measured by counting the number of infected colony cells on Petrifilm (3M Co., St. Paul, MN).

### 2.3. Colony direct PCR

After the panning, colonies of phage-infected TG1 were picked up at random as PCR templates. The gene inserts of 16 clones were amplified by PCR using the following primers: primer-156 (5'-CAACGTGAAAATTTATTCGC-3') and primer-158 (5'-GTAATACA ATTTCGTATGACG-3'), which anneal to the sequences of pCANTAB5E phagemid vector (GE Healthcare Biosciences AB, Uppsala, Sweden). The size of insert DNA sequence was analyzed by agarose gel electrophoresis.

### 2.4. Cell lines

Human mammary gland cell line 184A1 (American Type Culture Collection; ATCC, Manassas, VA) was maintained by MEGM Bullet Kit (Takara Bio, Shiga, JAPAN). Mammary gland-derived breast cancer cell line SKBR3 (ATCC) was maintained in McCoy's 5a plus 10% FBS. All cells were grown at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

### 2.5. 2D-DIGE analysis

Cell lysates were prepared from human mammary gland cell line 184A1 and mammary gland-derived breast cancer cell line SKBR3, and then solubilized with 7 M urea, 2 M thiourea, 4% CHAPS and 10 mM Tris-HCl (pH 8.5). The lysates were labeled at the ratio 50 µg protein: 400 pmol Cy3 or Cy5 protein labeling dye (GE Healthcare

Biosciences AB) in dimethylformamide according to the manufacturer's protocol. For first dimension separation, the labeled samples (each 50 µg) were combined and mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% Pharylmalyte (GE Healthcare Biosciences AB)) and applied to a 24-cm immobilized pH gradient gel strip (IPG-strip pH 5–6 NL). The samples for the spot-picking gel were prepared without labeling by Cy-dyes. For the second dimension separation, the IPG-strips were applied to SDS-PAGE gels (10% polyacrylamide and 2.7% N,N'-diallyltartardiamide gels). After electrophoresis, the gels were scanned with a laser fluorometer (Typhoon Trio, GE Healthcare Biosciences AB). The spot-picking gel was scanned after staining with Flamingo solution (Bio-Rad). Quantitative analysis of protein spots was carried out with Decyder-DIA software (GE Healthcare Biosciences AB). For the antigen spots of interest, spots of 1 × 1 mm in size were picked using an Eitan Spot Picker (GE Healthcare Biosciences AB). Proteins were extracted by solubilizing the picked gel pieces using 88 mM sodium periodate. Protein volumes were determined by BSA standard in Colloid Total Protein staining (Bio-Rad).

### 2.6. In-gel tryptic digestion

Spots of 1 mm × 1 mm in size were picked using an Eitan Spot Picker and digested with trypsin as described below. The gel pieces were then destained with 50% acetonitrile/50 mM NH<sub>4</sub>HCO<sub>3</sub> for 20 min twice, dehydrated with 75% acetonitrile for 20 min, and then dried using a centrifugal concentrator. Next, 5 µl of 20 µM/ml trypsin (Promega, Madison, WI) solution was added to each gel piece and incubated for 16 h at 37 °C. Three solutions were used to extract the resulting peptide mixtures from the gel pieces. First, 50 µl of 50% (v/v) acetonitrile in 1% (v/v) aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 5 min. Next, we collected the solution and added 80% (v/v) acetonitrile in 0.2% TFA. Finally, 100% acetonitrile was added for the last extraction. The peptides were dried and then resuspended in 10 µl of 0.1% TFA before being cleaned using ZipTip™ µC<sub>18</sub> pipette tips (Millipore, Billerica, MA). The tips were wetted with three washes in 50% acetonitrile and equilibrated with three washes in 0.1% TFA, then the peptides were aspirated 10 times to ensure binding to the column. The column and peptides were washed three times in 0.1% TFA before being eluted in 1 µl of 80% acetonitrile/0.2% TFA.

### 2.7. Mass spectrometry (MS) and database search

The tryptic digests (0.5 µl) were mixed with 0.6 µl α-cyano-4-hydroxy-trans-cinnamic acid saturated in 0.1% TFA and acetone (4:1 v/v). Each mixture was deposited onto a well of a 96-well target plate and then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS; autoXcell, Bruker Daltonics, Billerica, WI) in the Reflection mode. The mass axis was adjusted with calibration peptide (BRUKER DALTONICS) peaks (M/z 10479.1, 12968.6, or 24651.9) as lock masses. Bioinformatic databases were searched to identify the proteins based on the tryptic fragment sizes. The Mascot search engine (<http://www.matrixscience.com>) was initially used to query the entire theoretical tryptic peptide as well as SwissProt (<http://www.expasy.org/>), a public domain database provided by the Swiss Institute of Bioinformatics, Geneva, Switzerland). The search query assumed the following: (i) the peptides were monoisotopic (ii) methionine residues may be oxidized (iii) all cysteines are modified with iodoacetamide.

### 2.8. Phage ELISA using nitrocellulose membrane

Phage ELISA using scFv antibody-expressing phages was performed as previously described [22]. Briefly, phage-infected TG1 clones were picked, monocloned in a Bio-Dot Microfiltration Apparatus and scFv antibody-expressing phages propagated. The supernatants containing scFv antibody-expressing phages were incubated with immobilized proteins (~1 ng) extracted from 2D-DIGE spots. scFv antibody-expressing phages bound to 2D-DIGE spots were visualized using HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare Biosciences AB).

### 2.9. Immunohistochemical staining using scFv antibody-expressing phages

Human breast cancer and normal TMA (Super Bio Chips, Seoul, South Korea & Biomax, Rockville, MD) were deparaffinized in xylene and rehydrated in a graded series of ethanol. Heat-induced epitope retrieval was performed in while keeping Target Retrieval Solution pH 9 (Dako, Glostrup, Denmark) temperature following the manufacturer's instructions. Heat-induced epitope retrieval was performed while maintaining the Target Retrieval Solution pH 9 (Dako) at the desired temperature according to the manufacturer's instructions. After heat-induced epitope retrieval treatment, endogenous peroxidase was blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in TBS for 5 min followed by washing twice in TBS. TMA were incubated with 5% BSA blocking solution for 15 min. The slides were then incubated with the primary scFv antibody-expressing phages (10<sup>12</sup> CFU/ml) for 60 min. After washing three times with 0.05% TBST, each series of sections was incubated for 30 min with ENVISION + Dual Link (Dako), washed three times in TBST. The reaction products were rinsed twice with TBST, and then developed in liquid 3,3'-diaminobenzidine (Dako) for 3 min. After the development, sections were washed twice with distilled water, lightly

counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with resinous mounting medium. All procedures were performed using AutoStainer (Dako).

### 2.10. TMA Immunohistochemistry scoring

The optimized staining condition for breast tumor tissue microarray was determined based on the coexistence of both positive and negative cells in the same tissue sample. Signals were considered positive when reaction products were localized in the expected cellular component. The criteria for the staining were scored as follows: distribution score was scored as 0 (0%), 1 (1–50%), and 2 (51–100%) to indicate the percentage of positive cells in all tumor cells present in one tissue. The intensity of the signal (intensity score) was scored as 0 (no signal), 1 (weak), 2 (moderate) or 3 (marked). The total of the distribution score and intensity score was then summed into a total score (TS) of TS0 (sum = 0), TS1 (sum = 2), TS2 (sum = 3), and TS3 (sum = 4–5). Throughout this study, TS0 or TS1 was regarded as negative, whereas TS2 or TS3 was regarded as positive. Statview software was used in statistical analysis.

## 3. Results

### 3.1. Optimization of panning methods

To establish a method for the efficient isolation of antibodies against a small amount of protein antigen (nanogram-order or less) prepared from 2D-DIGE spots, 5000 ng, 50 ng or 0.5 ng of recombinant KDR proteins were first immobilized on a BIAcore sensor chip CM3<sup>®</sup> or on a nitrocellulose membrane using the Bio-Dot Microfiltration Apparatus<sup>®</sup>. Isolation of antibodies was assessed using a model phage library (anti-KDR scFv antibody-expressing phages: wild type phage = 1: 100) (Fig. 1). Enrichment of the desired clones in the output library was evaluated by analyzing the gene inserts of randomly-picked phage-infected TG1 cells by colony direct PCR. In the method using BIAcore<sup>®</sup>, enrichment was observed when 5000 ng of KDR was used for immobilization. By contrast, Membrane-based panning led to the successful enrichment of anti-KDR scFv antibodies from only 0.5 ng of KDR. These results demonstrated that membrane-based panning was suitable for the isolation of antibodies from very small amounts of antigen extracted from 2D-DIGE spot gel pieces.

### 3.2. 2D-DIGE analysis and identification of differentially expressed proteins

To identify breast tumor-related biomarker proteins and isolate monoclonal antibodies against them, we performed 2D-DIGE using

breast cancer cell lines SKBR3 and normal breast cell lines 184A1 (Fig. 2). Quantitative analysis showed that 21 spots displayed increased or decreased expression levels in the cancer cell line compared with the normal cell line. MALDI-TOF/MS analysis of the spots subsequently identified 16 different proteins (Table 1).

### 3.3. Isolation of antibodies against each 2D-DIGE spot from the non-immune scFv phage library

The amount of protein extracted from the gel pieces ranged from several tens of nanogram to a few micrograms (Table 1). Because the membrane-based panning method facilitates the isolation of antibodies from 0.5 ng of protein (Fig. 1), we reasoned that this method could be used to isolate antibodies from the small amounts of proteins extracted from 2D-DIGE spot gel pieces. Thus a portion of the extracted proteins were immobilized onto nitrocellulose membranes by means of a Bio-Dot Microfiltration Apparatus, and membrane-based panning was performed using the non-immune scFv phage library [22] (Table 2). The results from this panning showed that the output/input ratio of phage titer (titer of the recovered phage library after the panning/titer of phage library before the panning) after the fourth round of panning against all spots increased approximately 20-fold–4000-fold in comparison to that obtained from the first round of panning. This elevated output/input ratio indicated the enrichment of the antigen-binding scFv antibody clones. To isolate monoclonal scFv antibodies to each spot, a total of 60 clones were randomly picked from the 4th panning output phage library and binding of the monoclonal scFv antibody-expressing phages to each antigen was tested by phage ELISA. As a result, several scFv antibody clones binding to each of the 16 antigens were isolated (Table 2). The antigenic specificity of isolated scFv antibodies was investigated by dot blot using various proteins as antigens. Some of the isolated scFv antibodies bound specifically to the antigen protein, but not to the His-tagged caspase-8, His-tagged importin- $\beta$ , tumor necrosis factor receptor 1 (TNFR1)-Fc-chimera and KDR-Fc-chimera (data not shown). These results indicated the successful isolation of each spot-specific scFv antibody-expressing phages after only two weeks.

### 3.4. TMA analysis

The next stage in the process was to select the most valuable breast tumor-related biomarker proteins from a large number of

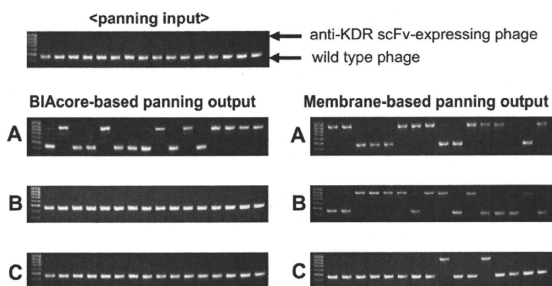
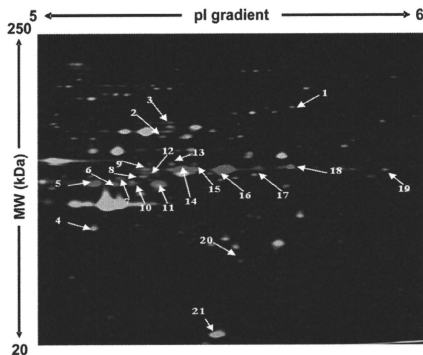


Fig. 1. Optimization of panning methods to isolate monoclonal antibodies from a very small amount of antigen. Model panning was performed using the BIAcore<sup>®</sup> or nitrocellulose membrane. The model library (anti-KDR scFv phage: wild type phage = 1: 100) was incubated with KDR ((A) 5000 ng, (B) 50 ng, (C) 0.5 ng) immobilized on a sensor chip or nitrocellulose membrane. The BIAcore-based panning method has been previously described [22]. After the binding step, the nitrocellulose membrane was washed ten times with TBST. The bound scFv antibody-expressing phages were eluted with triethylamine. The eluted scFv antibody-expressing phages were then incubated in log phase TG1 cells and individual TG1 clones were picked at random. Inserts of 16 phage clones were amplified by PCR. The gene sizes of inserts were analyzed by agarose gel electrophoresis.



**Fig. 2.** 2D-DIGE image of fluorescently labeled proteins from SKBR3 and 184A cell. Breast cancer cell line (SKBR3) and normal breast cell line (184A1) were labeled using cy3 and cy5, respectively. The protein samples were then subjected to 2D electrophoresis. Spots that were over- and under-expressed in mammary cancer cells relative to normal cells were colored red and green, respectively. Yellow color spots show no change in expression.

identified candidate proteins. To this end, we immunostained TMA slides with 189 cases of breast tumors and 15 cases of normal breast specimens using the isolated spot-specific scFv antibody-expressing phages and screened the promising candidate biomarker proteins in terms of the expression profile in breast tumor tissues and normal tissues (Table 3). The result of the expression profile analysis showed that SPATAS5, beta-actin variant, FLJ31438, PAK65, XRN1 and Jerky protein homolog-like were not expressed in

**Table 2**  
Enrichment and isolation of antibodies to 2D-DIGE spots from non-immune libraries.

Spot	Protein name	Output/Input Ratio ( $\times 10^{-3}$ )/round				The number of isolated mAb.
		1st	2nd	3rd	4th	
#1	splicing factor YT521-B	6	7	16	480	4
#2	IkappaB $\alpha$ R	6	7	15	500	3
#3	SPATAS5	5	6	32	860	2
#4	skin aspartic protease	5	6	5	24	1
#5	beta actin variant	7	11	17	480	1
#6	TRAIL-R2	6	7	25	420	5
#7	Cytokeratin 18	5	11	62	260	4
#8	TRAIL-R2	5	27	41	1500	5
#9	RREB1	8	9	14	370	7
#10	Cytokeratin 7	6	7	3	2200	5
#11	Cytokeratin 18	6	8	15	84	2
#12	Cytokeratin 7	10	11	13	94	2
#13	FLJ31438	7	9	32	80	6
#14	Cytokeratin 7	4	7	46	280	5
#15	PAK65	7	11	51	580	9
#16	Cytokeratin 8	8	7	16	4100	6
#17	Cytokeratin 8	5	12	33	240	2
#18	XRN1	6	20	18	200	1
#19	Jerky protein homolog-like	7	10	49	940	3
#20	Eph receptor A10	8	6	57	3000	2
#21	Glutathione S-transferase P	7	8	110	1900	2

normal and breast cancer tissue at all. By contrast, TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were highly and specifically expressed (Fig. 3) in 63, 73 and 49% of breast tumor cases respectively, while the existing-breast cancer marker, Her-2, was expressed in 28% of breast tumor cases (Table 3). Thus, the relationship between the expression of each antigen and the Her-2 expression profile was analyzed. The level of expression of TRAIL-R2, Cytokeratin 8 and Eph receptor A10 in Her-2 positive cases were 77, 77 and 62%, and in Her-2 negative cases were 57, 67 and 44%, respectively (Table 4). Furthermore, the relationship between the expression of each antigen and clinical stage was analyzed in 187 of the 189 cases where all the clinical data was available. The level of expression of Cytokeratin 8 and Eph receptor A10 increased with progression of clinical symptoms (Table 5).

**Table 1**  
Identification of 2D-DIGE spots by MALDI-TOF/MS.

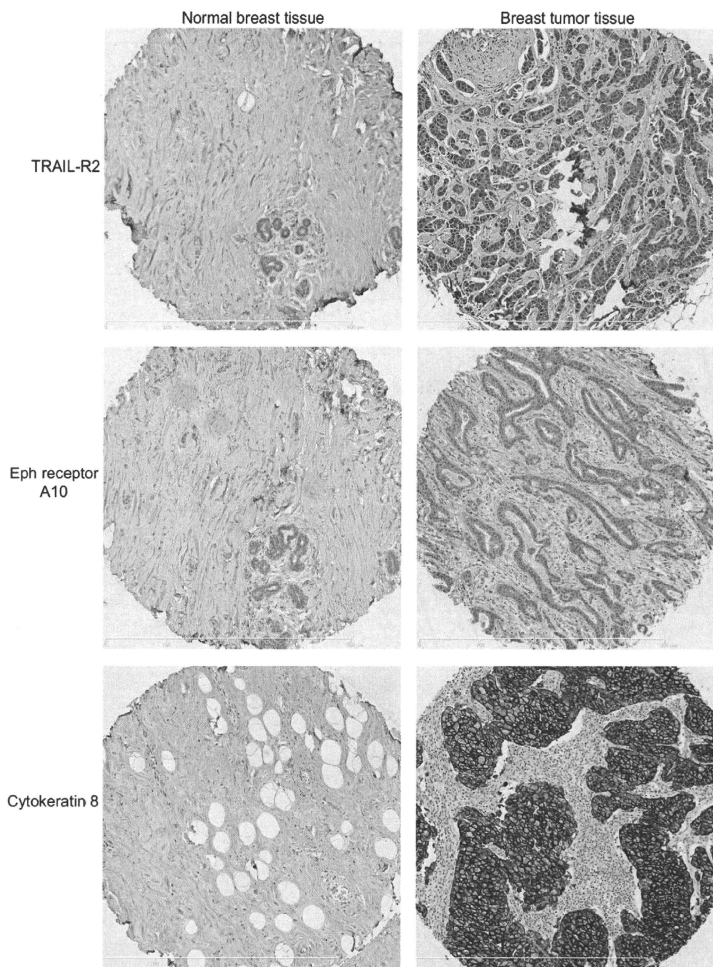
Spot	Protein name	Accession number	MW (kDa)	pI	Protein volume (ng)	Expression ratio [cancer/normal] (fold)
#1	splicing factor YT521-B	Q96MU7	85	5.9	119	6
#2	IkappaB $\alpha$ R	Q96HA7	63	5.5	104	6
#3	SPATAS5	C9JF97	76	5.6	94	7
#4	skin aspartic protease	Q53RT3	37	5.3	610	0.1
#5	beta actin variant	P60709	42	5.3	99	15
#6	TRAIL-R2	O14763	48	5.4	100	18
#7	Cytokeratin-18	P05783	48	5.3	99	12
#8	TRAIL-R2	O14763	48	5.4	95	16
#9	RREB1	Q92766	52	5.3	109	10
#10	Cytokeratin-7	P08729	51	5.4	126	23
#11	Cytokeratin-18	P05783	48	5.3	497	13
#12	Cytokeratin-7	P08729	51	5.4	122	24
#13	FLJ31438	Q96N41	53	5.5	126	35
#14	Cytokeratin-7	P08729	51	5.4	406	36
#15	PAK65	Q13177	55	5.7	677	8
#16	Cytokeratin 8	P05787	54	5.5	694	32
#17	Cytokeratin 8	P05787	54	5.5	1143	72
#18	XRN1	Q8J2H2	54	5.8	353	8
#19	Jerky protein homolog-like	Q9Y4A0	51	6.0	130	22
#20	Eph receptor A10	Q5JZY3	32	5.7	119	9
#21	Glutathione S-transferase P	P09211	23	5.4	119	0.02

#### 4. Discussion

Here, we aimed to develop a method of efficiently screening tumor-related biomarker proteins by proteome analysis. In

**Table 3**  
Positive rate of identified proteins in breast cancer and normal breast tissues.

Protein name	Positive rate of antigens	
	Normal breast tissues	Breast cancer tissues
Her-2	0/15 (0%)	53/189 (28%)
IkappaB $\alpha$ R	3/15 (20%)	22/189 (12%)
SPATAS5	0/15 (0%)	0/189 (0%)
beta actin variant	0/15 (0%)	0/189 (0%)
TRAIL-R2	0/15 (0%)	119/189 (63%)
RREB1	1/15 (7%)	83/189 (44%)
FLJ31438	0/15 (0%)	0/189 (0%)
PAK65	0/15 (0%)	0/189 (0%)
Cytokeratin 8	0/15 (0%)	137/189 (73%)
XRN1	0/15 (0%)	0/189 (0%)
Jerky protein homolog-like	0/15 (0%)	0/189 (0%)
Eph receptor A10	0/15 (0%)	93/189 (49%)



**Fig. 3.** Immunohistochemical staining of breast tumor and normal breast tissue microarray by scFv antibody-expressing phages. Typical images of breast cancer and normal breast tissue microarray stained by using scFv antibody-expressing phages to TRAIL-R2, Eph receptor A10 and Cytokeratin 8 are shown. Left panels are normal breast tissues and right panels are breast tumors. The tissue microarrays were counterstained by hematoxylin.

particular, we attempted to establish a means of isolating specific antibodies directly from small amounts of differentially expressed proteins obtained via 2D-DIGE analysis. To achieve this, we focused on a non-immune scFv phage library. Because the non-immune naïve scFv phage library has a huge repertoire of scFv on the surface of the phages, monoclonal antibodies to every antigen could be effectively isolated *in vitro*. Generally the diversity of the CDR3 domain, which is important for antigen-binding specificity, is

estimated to be approximately twenty million [23]. Thus we reasoned that our previously constructed library, containing  $2.4 \times 10^9$  scFv variants, has almost equal potential as the murine or human immune system [22]. Initially, in order to isolate monoclonal antibodies against very small amounts of antigen (hundreds of nanograms) recovered from the spots of 2D-DIGE analysis, we attempted to optimize the panning method using either a BIAcore® or nitrocellulose membrane. In the method using BIAcore®, the

**Table 4**  
Positive rate of identified proteins in Her-2 positive and Her-2 negative cases.

Protein name	Positive rate of antigens in Her-2	
	Positive cases	Negative cases
TRAIL-R2	41/53 (77%)	78/136 (57%)
Cytokeratin 8	41/53 (77%)	91/136 (67%)
Eph receptor A10	33/53 (62%)	60/136 (44%)
TRAIL-R2 or Eph receptor A10	46/53 (87%)	100/136 (74%)

enrichment of the desired clones was observed when immobilizing 5000 ng of KDR. By contrast, membrane-based panning led to the successful enrichment of clones from only 0.5 ng of KDR (Fig. 1). BIAcore-based panning has been recognized to be an effective method because the interaction of an antigen and a scFv antibody can be monitored in real time and the operation can be automated [24,25]. However, our results suggest that BIAcore® is inefficient for immobilizing very small amounts of antigen. This is because antigen immobilization using the BIAcore procedure requires a chemical coupling reaction with the surface of the sensor chip. In contrast, the membrane-based panning method is suitable for the isolation of antibodies against very small amounts of antigens. The suitability of this procedure when handling such small amounts of proteins presumably arises from the high efficiency of adsorption of antigens by the nitrocellulose membrane. These results show that monoclonal antibodies can be created from small amounts of proteins recovered from 2D-DIGE spots.

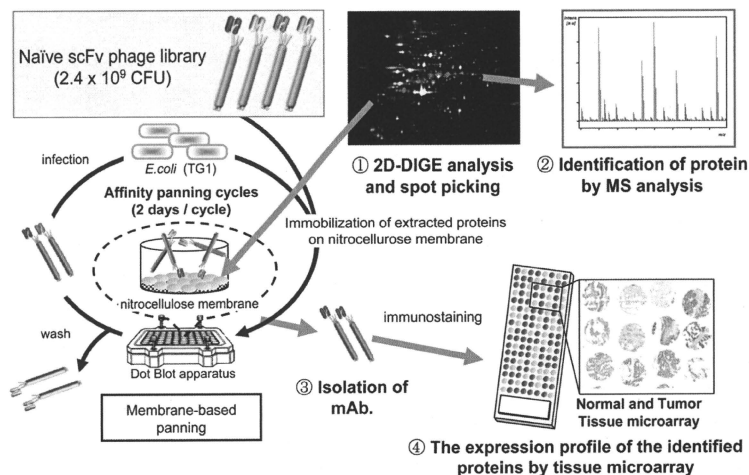
In breast cancer patients, the antibody targeting human epidermal growth factor receptor II (Her-2), is an effective drug [26,27]. However, because this receptor is over-expressed in only ~25% of breast cancer patients, anti-Her-2 antibody therapy is ineffective in ~75% of cases. Furthermore, approximately 30% of Her-2 over-expressed patients that received anti-Her-2 antibody therapy became tolerant [28–30]. Thus, we applied our antibody

**Table 5**  
Positive rate of identified proteins in clinical stage.

Protein name	Positive rate of antigens in clinical stage		
	Stage I	Stage II	Stage III
Her-2	6/14 (43%)	17/87 (20%)	30/86 (35%)
TRAIL-R2	11/14 (79%)	51/87 (59%)	55/86 (64%)
Cytokeratin 8*	7/14 (50%)	58/87 (67%)	71/86 (83%)
Eph receptor A10*	4/14 (29%)	42/87 (48%)	47/86 (55%)

Man Whitney U test \*P < 0.05

proteomics system to breast cancer samples for identification of the proteins to replace Her-2 as suitable therapeutic targets. Initially, 21 differentially expressed proteins between SKBR3 and 184A1 cells were found by 2D-DIGE analysis and 16 different proteins were identified by MALDI-TOF/MS. Four of the identified proteins were present in more than one spot i.e., TRAIL-R2 (spot 6, 8), Cytokeratin 18 (spot 7, 11), Cytokeratin 8 (spot 16, 17) and Cytokeratin 7 (spot 10, 12, 14). These proteins presumably display different pI and MW values due to posttranslational modification. Next, membrane-based panning against these spots was performed, and the output/input ratio of phage titer after the fourth round of panning increased from approximately 20-fold–4000-fold in comparison to that after the first round of panning. Moreover, we screened scFv antibody-expressing phages binding to each spot protein by phage ELISA and obtained each spot-specific scFv antibodies from all spots after approximately two weeks. Finally, it was necessary to select the most valuable proteins from a large number of differentially expressed proteins in breast cancer cells. Using the isolated spot-specific scFv antibody-expressing phages, we immunostained a TMA with 189 cases of breast cancer tissue and 15 samples of normal tissue. SPATAS, Beta actin, FLJ31438, PAK65 and XRN1 were not detected in either the tumor tissue or normal tissue. Thus, these proteins may have been derived from cell lines used in the



**Fig. 4.** Schematic illustration of the antibody proteomics system. Antibody proteomics system is an efficient method for screening tumor-related biomarker proteins. Because this system involves the direct isolation of monoclonal antibodies from 2D-DIGE spots without preparation of recombinant proteins, it enables the discovery and validation of tumor-related biomarker proteins by TMA analysis using the isolated scFv antibody-expressing phages.

proteome analysis or the antibodies against these proteins may not detect the antigen on formalin-fixed paraffin-embedded tissues. By contrast, TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were specifically-expressed in over 40% of breast cancer tissues. We confirmed the immunohistochemical staining image generated by scFv antibody-expressing phages displayed a similar pattern to that generated by IgG type commercial antibody (data not shown). Interestingly, the expression rates of TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were higher than the existing breast cancer marker, Her-2 (only about 25%). Moreover, the expression rates of TRAIL-R2 and Eph receptor A10 (cell membrane proteins) in Her-2 negative cases were over 40% and in Her-2 positive cases over 60%. This data indicates that TRAIL-R2 and Eph receptor A10 are promising alternative target candidates for anti-Her-2 antibody therapy ineffective patients, at least in terms of the expression profile. Further work is required to analyze the function of these proteins in more detail. Furthermore, by checking antigen expression profiles against clinical information, the expression rate of Cytokeratin 8 and Eph receptor A10 was found to have increased during progression of the clinical symptoms. These observations indicate that Cytokeratin 8 and Eph receptor A10 are promising diagnostic marker candidates for assessing the aggressiveness of breast cancer.

Recently, an anti-TRAIL-R2 antibody has been developed as an anticancer drug [31–33]. Moreover, Cytokeratin 8 has gained considerable attention as a cancer aggressiveness diagnostic marker [34–36]. These results demonstrate that this technology is able to select well-known drug-target markers (i.e., TRAIL-R2) and diagnostic markers (i.e., Cytokeratin 8) as well as unknown biomarker protein candidates (Eph receptor A10) from a large variety of differentially expressed proteins in cancer cells.

Our method employs a set of techniques for efficiently identifying biomarker candidates. Specifically, the method entails: 1) searching for differentially expressed proteins in disease samples, 2) identification of the proteins, 3) high throughput isolation of monoclonal antibodies against the proteins using a naïve scFv phage library, and 4) validation of the proteins by TMA analysis. This methodology is referred to as an "antibody proteomics system" (Fig. 4). We believe that the proteins identified using this approach will contribute to the drug development process. Indeed, the antibody proteomics system could become a platform technology for seeking tumor-related biomarker proteins by a proteomics-based approach.

## 5. Conclusions

In this study, we established the antibody proteomics system for efficiently screening and validating tumor-related biomarker proteins of interest by isolating specific antibodies directly from small amounts of proteins obtained via 2D-DIGE analysis. Applying this technique to the identification of breast tumor-related biomarker proteins, the expressions of Eph receptor A10, TRAIL-R2 and Cytokeratin 8 in breast tumor tissues were successfully validated from a large number of candidates. These results demonstrate that our original technology is an efficient and useful method for screening tumor-related biomarker proteins. Moreover, Eph receptor A10, TRAIL-R2 and Cytokeratin 8 identified in this study are promising breast tumor biomarkers for drug development.

## Acknowledgement

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## Appendix

Figure with essential color discrimination. Figs. 2–4 in this article have parts that are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.09.030.

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