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創薬基盤推進研究事業

抗体プロテオミクス技術を駆使した
悪性中皮腫関連バイオマーカーの探索と創薬への展開

平成 22 年度　総括研究報告書

研究代表者　長野一也

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研究代表者　長野一也　　独立行政法人医薬基盤研究所　創薬基盤研究部

研究要旨

悪性中皮腫は、1970年頃に頻用されたアスベストの曝露を主要因とする疾患であり、5年生存率は3.7%と極めて予後不良である。また本疾患は、この曝露から40年程遅れて発症することから、現在患者数は急増している。以上の背景から、悪性中皮腫に対する有用な診断法・治療法の開発は国際的緊急課題であるものの、これまで悪性中皮腫の分子マーカーはみつかっていない。そこで本研究では、独自に確立した創薬バイオマーカー蛋白質の迅速探索法である「抗体プロテオミクス技術」により、悪性中皮腫関連マーカーを探索し、病態の解明、並びに有用な診断法・治療戦略の確立に資する知見をえることを目的とする。

これを達成するため、本年度は、悪性中皮腫/正常中皮細胞に発現している蛋白質の変動解析により、悪性中皮腫関連マーカー候補を同定し、これらのバリデーションに必要なモノクローナル抗体をファージ抗体ライブラリ法により作製した。さらに、本疾患の治療薬として汎用されているシスプラチンに対する感受性マーカーの同定も目指し、発現変動を解析するためのソースに用いる高感受性株と低感受性株を多くの悪性中皮腫細胞株の中からスクリーニング･決定した。

A. 研究目的

現在、疾患マーカーや創薬ターゲットなどの創薬バイオマーカー蛋白質の同定は、画期的医薬に直結することから、プロテオミクス研究を中心に世界規模で進められている。しかしながら、当該研究領域からこれまでに医薬品開発にまで至った例は殆どない。それはプロテオーム解析から見出される候補蛋白質の数が多く、この中から有用な分子を絞り込むための基盤技術が未成熟であることに起因している。本観点から申請者らは、プロテオーム解析から直接同定・回収される微量な抗原蛋白質を、吸着力に優れるニトロセルロース膜に固相することによって、ファージ抗体ライブラリの中から短期間かつ網羅的にモノクローナル抗体を作製可能な方法論を構築した。これにより、取得した抗体で組織アレイ解析し、多症例のがん組織が有する臨床情報と各候補蛋白質の発現分布との相関から疾患マーカーや創薬ターゲットなどの創薬バイオマーカー蛋白質となりうる有用な分子を絞り込むことが可能となった。そこで本研究では、独自に確立した創薬バイオマーカー蛋白質の効率的探索法である「抗体プロテオミクス技術」を駆使し、未だ本邦の死亡率第一位のがんの中でも、5年生存率が僅か3.7%と極めて予後不良な悪性中皮腫に応用することで、悪性中皮腫関連マーカー蛋白質を探索し、疾患の診断法・治療戦略の確立に役立つ情報を得ると共に、創薬への展開を目指すことを

目的とする。

悪性中皮腫は、1970年頃に頻用されたアスベストの曝露を主要因とする疾患であり、曝露から40年程遅れて発症することから、現在患者数は急増している。一方で、本疾患に対する有用な診断・治療法はなく、分子病態すら殆ど明らかにされていないことから、厚生労働行政にとって政策対応を必要とする疾患である。従って本研究によって得られる成果は、厚生労働行政にとって今後政策的な対応を必要とする悪性中皮腫に対して、基礎研究・応用研究の両者を発展させると共に、国民の健康増進や医療費削減、産業界の競争力向上等に貢献するものと期待される。

このような背景･目的のもとで1年目の研究を実施し、悪性中皮腫細胞と正常中皮細胞に発現する蛋白質の変動解析と同定、並びにファージ抗体ライブラリによって各々の抗体を計画通り取得した。さらに、悪性中皮腫治療に汎用されるシスプラチン耐性マーカーの同定も目指し、各悪性中皮腫細胞株のシスプラチンの感受性試験によって、高感受性株と低感受性株を同定したのでこれら研究成果について報告する。

B. 研究方法

細胞培養

悪性中皮腫細胞株（H28、H2052、H2452、H226、MSTO221H）はATCCより購入したものを用いた。培養には、10% FCS/RPMI1640を用い、いずれも継代培養してサブコンフルエント状態のものを実験に供した。Human Mesothelial Cells（HMC）はZen-Bioより購入したものを用いた。培養には、Mesothelial Cell Growth Medium（Zen-Bio）を用い、継代培養してサブコンフルエント状態のものを実験に供した。

Two dimensional differential in-gel electrophoresis (2D-DIGE) 解析

通常培養環境下で培養したH28、HMCをサンプルとして用いた。サンプル各50 μ gをそれぞれ400 pmol のラベル化試薬 cy2 、 cy3 、 cy5 （GE Healthcare）と氷上で30分間反応させ、その後10 mM Lysineを加え、氷上で10分間静置して反応を停止させた。標識されたサンプルを全て混合し、sample buffer（2% DTT、2% pharmalyte (GE Healthcare)、7 M urea、2 M thiourea、4% CHAPS）で450 μ lにメスアップした。一方、蛋白質を回収するためのピックゲル用に、ラベル化試薬で標識していないサンプルも同様に混合調製した。等電点泳動用の専用ホルダーにサンプルを注入して、IPG-gel（pI 4-7）ストリップ（GE Healthcare）を入れ、 oil を重層した。ETTAN IPGPhor （GE Healthcare）を用いて、プレ膨潤を10時間行い、等電点電気泳動を行った。泳動終了後、IPG-gelを平衡化buffer A （Tris-HCl（pH 6.8）、6 M urea、30% glycerol、2% SDS、0.002% BPB、10 mg/ml DTT）と平衡化buffer B (Tris-HCl (pH 6.8)、6 M urea、30% glycerol、2% SDS、0.002% BPB、25 mg/ml iodoacetamide（Sigma））に浸し、各15分間平衡化を行った。二次元目のSDS-PAGEを行うため、ゲル溶解が可能なSDS-PAGE用ゲル（10% polyacrylamide and 2.7% N,N'-diallyl -tartardiamide gels）にIPG-gelスリップをセットた。アガロースで封入後、 Ettan Daltsix Electrophoresis System（GE Healthcare）を用いて、2次元電気泳動を行った。ピック用ゲルはDeep Purple Total Protein Stainを用いて一晩染色し、脱色液により脱色を行った。解析には、Typhoon scanner、Ettan DIGEを使用し、スポットピックにはEttan Spot Picker(GE Healthcare)を使用した。抗体作製用の蛋白質抽出には、88 mM $NaIO_4$を用いて室温で30分インキュベーションし、ゲルを溶解することで蛋白質を抽出した。

MS解析

ゲル片に 100 µl の脱色液（25 mM ammonium bicarbonate （ Nacalai Tesque ） / 50% acetonitolile（Nacalai Tesque））を加え、室温で 10 分振盪させた後、脱色液を取り除くことで脱色を行った。続いて 200 µl の acetonitrile を加え、ゲル片が白濁した後取り除き、遠心濃縮器によって乾燥させることで脱水を行った。脱水したゲル片に 5 µl の trypsin 溶液（20 µl/ml trypsin（Promega） / 50 mM ammonium bicarbonate）を加え、37℃で 16 時間反応させることで、ゲル内の蛋白質を消化した。消化後、ゲル片に抽出液（1 回目は 50 µl の 50% acetonitrile / 5% TFA 溶液、2 回目は 50 µl の 80% acetonitrile / 5% TFA 溶液、3 回目は 50 µl の 100 % acetonitrile）を加え、3 分間ソニケーションし、更に 30 分間ボルテックスした後の抽出液を回収するという操作を 3 回行うことでペプチドを抽出した。このペプチド抽出液を遠心濃縮器によって濃縮し、これをサンプル溶液とした。サンプル溶液 5 µl を nano-LC (EASY-nLC (Bruker Daltonics)) にて分離し、各画分のペプチドを ESI-Q-TOF MS (maxis (Bruker Daltonics))にて配列の決定並びに、蛋白質同定を行った。なお、ペプチドの同定には、メチオニン残基の酸化、iodoacetamide によるシステイン残基のカルバミドメチル化を考慮した。

ナイーブファージ抗体ライブラリの作製

ナイーブファージ抗体ライブラリ遺伝子を組み込んだファージミドベクターを導入した大腸菌 TG1 株に 50 µg/ml ampicillin、2% glucose 含有 2YT 培地を加えて 250 rpm、37℃で OD_{600} = 0.3～0.6 まで培養した。M13KO7 ヘルパーファージ（Invitrogen）を添加し、110 rpm、37 ℃で 30 分間、250 rpm、37 ℃で 30 分間培養後、2,000 rpm で 10 分間遠心し、得られたペレットに対して 100 µg/ml ampicillin、50 µg/ml kanamycin 含有 2YT 培地を添加して 6 時間培養した。4 ℃、2,000 rpm で 10 分間、更に 10,000 rpm で 15 分間遠心し、回収した上清に氷冷した 20% PEG-6000、2.5 M NaCl を 1/5 volume 加え、激しく混和して氷上で 1 時間静置した。15,000 rpm で 10 分間遠心して得られたペレットを NTE buffer（100 mM NaCl、10 mM Tris、1 mM EDTA）に懸濁し、0.45 µm の Millex-HV フィルター (Millipore)を用いてろ過し、scFv 提示ファージとして回収した。

メンブランパンニング

ドットブロット装置（Bio-Dot Microfiltration Apparatus, BioRad）に、あらかじめ TBS に浸したニトロセルロース膜を装着し、可溶化ゲルから回収した各スポット由来の蛋白質溶液 1 µl を TBS にて 100 倍希釈した全量を各 well に添加し、メンブレンに固相化した。blocking buffer（10% skim milk + 25% glycerol）を 200 µl/well 添加して、室温で 1 時間静置してブロッキングした。scFv 提示ファージ溶液と blocking buffer とを 9 : 1 で混合し 4 ℃で 1 時間静置してブロッキングしたものを input とし、100 µl/well 添加して、室温で 3 時間緩やかに振盪した。0.1% Tween 20/TBS (0.1%TBST)で 10 回と TBS で 1 回洗浄後、100 mM triethylamine を 100 µl 添加して、室温で 30 分間静置した。output ファージ溶液を回収し、それらに 50 µl の Tris-HCl（pH 8.0）を加えて中和した。また、input ファージ及び output ファージの一部を用いてタイターを測定した。output ファージは大腸菌 TG1 株に感染させ、増殖させて上記のファージ作製法に準じてファージを産出し、再度同様のパンニング操作を行ったものを 2nd、3rd パンニング output とした。

Dot Blot ELISA によるスクリーニング

パンニング後に回収したファージを大腸菌TG1に感染させ、生じたコロニーを96 wellプレートにピックアップした。各wellがOD_{600} = 0.3～0.6に達するまで培養した後、100 μg/ml ampicillin、2% glucose含有2YT培地で10倍希釈したM13KO7ヘルパーファージ溶液を20 μl/wellで添加した。37 ℃で1時間静置培養した後、2,000 rpmで10分間遠心し、上清を除去した。100 μg/ml ampicillin、50 μg/ml kanamycin含有2YT培地を200 μl加えて37 ℃で一晩培養し、2,000 rpmで10分間遠心し、回収した上清を以下のスクリーニング実験に供した。一方で2D-DIGE解析から得られた抽出蛋白質をBio-Dot Microfiltration Apparatusを用い、TBSに浸したニトロセルロース膜上に固相化した。各wellにblocking buffer（10% skim milk & 25% glycerol）を200 μlずつ添加し、室温で2時間静置してブロッキングを行った。TBSで1回洗浄後、blocking bufferで希釈した精製ファージを200 μl/well添加し、室温で2時間静置した。0.1%TBSTとTBSで5回洗浄後、blocking bufferで1,000倍希釈したHRP/anti-M13 monoclonal antibodyを200 μl/well添加した。0.1%TBSTとTBSで3回洗浄後、メンブレンをECL plus Western Blotting Detection System（GE Healthcare）で処理し、LAS-3000を使用して検出・撮影した。

シスプラチン感受性試験

各悪性中皮腫細胞株を96 wellプレートに、5 x 10^3 cells/well播種し、一晩培養した。翌日、各濃度のシスプラチンを添加し、24時間後のViabilityをWST-8 assayにより評価した。

C. 研究結果

C-1. 悪性中皮腫細胞と正常中皮細胞に発現している蛋白質の変動解析

悪性中皮腫関連マーカーを探索するため、まず初代正常中皮細胞HMCに比べて、悪性中皮腫細胞株H28で発現変動している蛋白質の同定を試みた。H28とHMCの細胞溶解液を異なる蛍光色素でラベルし、等電点と分子量の違いにより2次元に蛋白質を展開した。各蛋白質スポットの蛍光強度の差違を解析した結果、1.5倍以上発現変動していた15スポットを見出すことができた (Fig. 1)。

C-2. 発現変動蛋白質の同定

上記で見出した15スポットを2D-DIGEのゲルからピックし、トリプシン消化後、LC-TOF/MSにより蛋白質の同定を試みた。MS/MS解析の結果、全ての発現変動蛋白質を同定し、14種類の悪性中皮腫特異的候補蛋白質を見出した (Table 1)。

C-3.メンブランパンニング法による発現変動蛋白質に対する抗体作製

C-1.で見出した15スポットを別途ピックし、過ヨウ素酸で溶解して抗原を調製した。まず、3種類の候補蛋白質に対してナイーブファージ抗体ライブラリを利用して、抗体の作製を試みた。4回のパンニングにより、目的抗原に親和性を有するクローンを100-1,000倍濃縮することができた (Fig. 2)。そこで、4^{th}パンニングのoutputをモノクローン化し、Dot Blot ELISAによりスクリーニングした。その結果、全ての候補蛋白質に対して、結合性を有する複数のクローンを単離することに成功した (Fig. 3)。

今後は、これらの抗体の特異性を評価したうえで、数多くの悪性中皮腫組織や正常中皮組織が搭載された組織マイクロアレイを免疫染色し、各抗原の発現プロファイルと各検体の臨床情報との相関解析によってバリデーションを試みる。

C-4. 悪性中皮腫細胞株のシスプラチン感受性試験

悪性中皮腫の治療薬としては、シスプラチンが汎用されているが、その奏功率は約20%と低い。そこでシスプラチン感受性症例を予め判別可能なマーカーの探索を目的に、高感受性細胞と低感受性細胞に発現している蛋白質の変動解析を試みる。本年度はまず悪性中皮腫細胞株の感受性をスクリーニングした。

各悪性中皮腫細胞株（H28、H2052、H2452、H226、MSTO221H）に各濃度のシスプラチンを添加し、各細胞への感受性をWST-8 assayにより評価した結果、H28が最も感受性が低く、H2052が最も高いことが判明した (Fig. 4)。今後はH28とH2052で発現変動している蛋白質を見出し、その中からシスプラチンの感受性に関与している蛋白質の同定を目指す。

D. 考察

C. 研究結果の欄に記載。

E. 結論

当該研究では、悪性中皮腫関連マーカーの探索を目的として、本年度は以下の知見を得た。

- 悪性中皮腫細胞株H28と初代正常中皮細胞HMCの2D-DIGE解析により、1.5倍以上発現変動している14種類の蛋白質を見出し、全て同定した。
- 上記の発現変動蛋白質に対して、ナイーブファージ抗体ライブラリによって抗体作製を試みた結果、抗原に結合性を有する複数のクローンを得た。
- シスプラチン感受性マーカーの探索も合わせて実施するため、各悪性中皮腫細胞株のシスプラチン感受性をスクリーニングしたところ、H28が最も感受性が低く、H2052が最も高いことを明らかとした。

F. 健康危険情報

該当なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

① 特許取得

無し

② 実用新案登録

無し

③ その他

無し

尚、下記に本研究の協力研究者を列挙する。

山下琢矢	独立行政法人医薬基盤研究所
矢野聖二	金沢大学がん研究所
常山幸一	富山大学大学院医学薬学研究部
堤　康央	大阪大学大学院薬学研究科
角田慎一	独立行政法人医薬基盤研究所
鎌田春彦	独立行政法人医薬基盤研究所
阿部康弘	独立行政法人医薬基盤研究所
森功美子	独立行政法人医薬基盤研究所
井上雅己	独立行政法人医薬基盤研究所
金崎聡一郎	独立行政法人医薬基盤研究所
古屋　剛	独立行政法人医薬基盤研究所
前田祐香	独立行政法人医薬基盤研究所

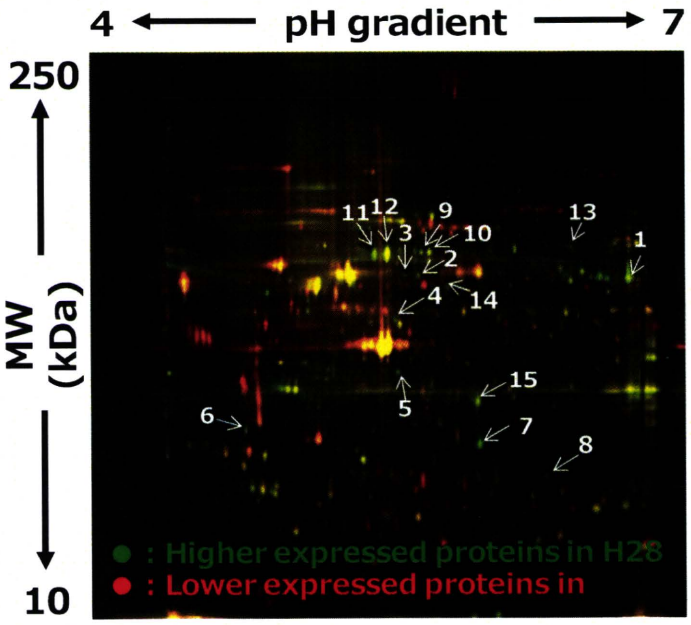

Fig. 1 2D-DIGE image of fluorescently labeled proteins derived from H28 and HMEC.
Proteins derived from H28 and HMEC were labeled with cy3 and cy5, and then separated by IEF-PAGE and SDS-PAGE. The spots indicated by allows are identified proteins by ESI-Q-TOF MS analysis.

Table 1 Identification of mesothelioma related proteins by ESI-Q-TOF MS

Spot #	Protein name	Expression raio (H28 / HMC)
1	Glucose-6-phosphate 1-dehydrogenase	6.0
2	FK506-binding protein 4	2.7
3	FK506-binding protein 4	2.7
4	Heterogeneous nuclear ribonucleoprotein F	3.5
5	Glutaredoxin 3	3.3
6	Proliferating cell nuclear antigen	3.0
7	Annexin A4	6.5
8	Glutathione tranceferase omega-1	1.8
9	T-complex protein 1 subunit ε	2.5
10	T-complex protein 1 subunit δ	2.2
11	60kDa heat shock protein	3.9
12	Spectrin bata chain, brain 1	3.4
13	Keratin, type Ⅰ cytoskeletal 10	3.4
14	Keratin, type Ⅱ cytoskeletal 8	2.6
15	L-lactate dehydrogenase B chain	1.8

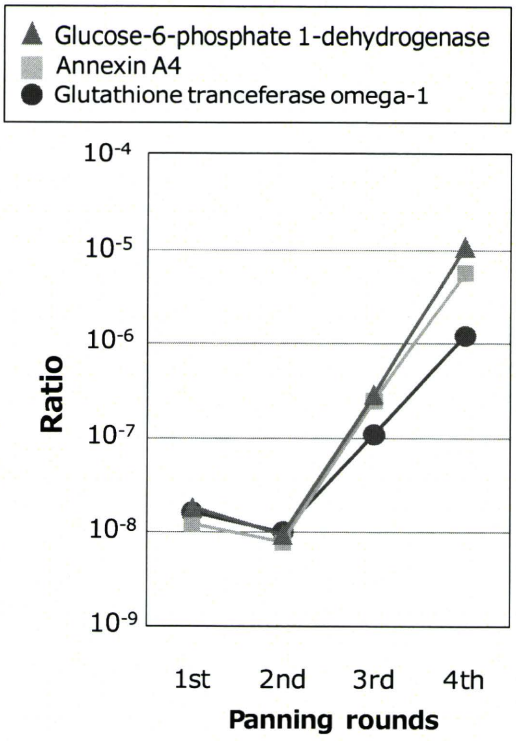

Fig. 2 Enrichment of Antibodies to mesothelioma related candidate proteins by membra ne panning

Enrichment of the desired clones was performed by membrane panning. The ratio of phage tit er at each panning round was plotted. The ratio was calculated as follows: (titer of the output phage) / (titer of the input phage).

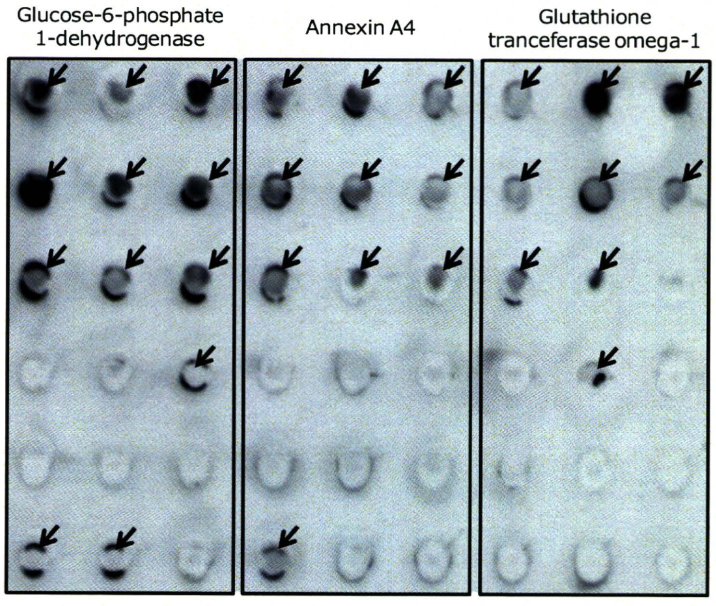

Fig. 3 Screening of antibody against mesothelioma related candidate proteins by Dot Bl ot ELISA

After the fourth panning, the binding properties of the selected phage clones were analyzed by Dot Blot ELISA. The positive clones were indicated by allows.

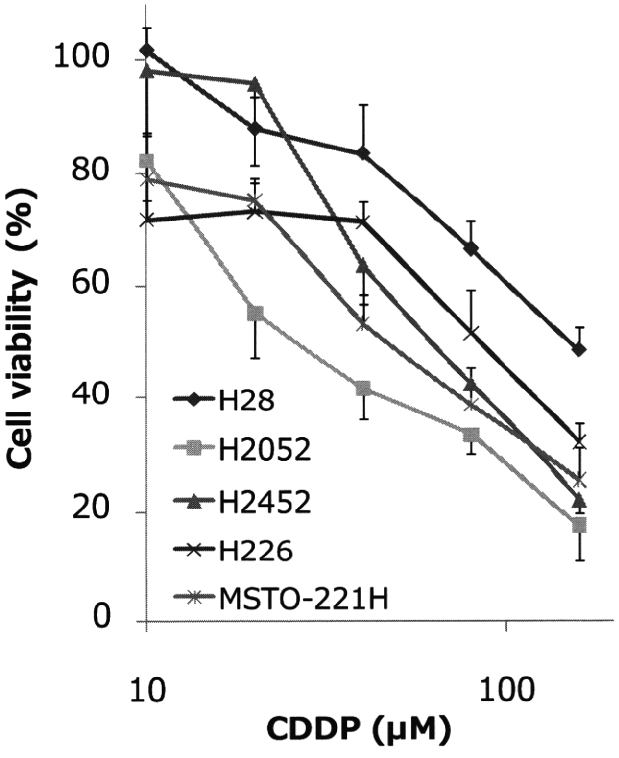

Fig. 4 Cytotoxicity analysis for cisplatin in mesothelioma cell lines
Mesothelioma cell lines, H28, H2052, H2452, H226 and MSTO-221H were incubated with vario us concentration of cisplatin for 24 hrs. Cytotoxicity of mesothelioma cells was evaluated by W ST-8 assay.

研究成果の刊行に関する一覧表

雑誌

著者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshida Y., Yamashita T., Nagano K., Imai S., Nabeshi H., Yoshikawa T., Yoshioka Y., Abe Y., Kamada H., Tsutsumi Y., Tsunoda S.	Limited expression of reticulocalbin-1 in lymphatic endothelial cells in lung tumor but not in normal lung.	Biochem. Biophys. Res. Commun.	405(4)	610-614	2011
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Limited expression of reticulocalbin-1 in lymphatic endothelial cells in lung tumor but not in normal lung

Yasunobu Yoshida [a,b,1], Takuya Yamashita [a,c,1], Kazuya Nagano [a,1], Sunao Imai [a], Hiromi Nabeshi [a,c], Tomoaki Yoshikawa [a,c], Yasuo Yoshioka [a,c,d], Yasuhiro Abe [a], Haruhiko Kamada [a,d], Yasuo Tsutsumi [a,c,d], Shin-ichi Tsunoda [a,b,d,*]

[a] Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[b] Department of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Department of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[d] The Center of Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Lymphatic endothelial cells in tumors (T-LECs) are considered to have different characteristics from LECs in non-tumor tissues (N-LECs). However, differences between the two types have not been well analyzed at molecular level. In this report, we performed differential proteome analysis of T-LEC and N-LEC models prepared by cultivation of LECs in tumor conditioned medium. By expression profiling of identified proteins using tissue microarrays, reticulocalbin-1 was found to be expressed in clinical specimen-derived T-LECs and lung cancer cells but not N-LECs. It is suggested that reticulocalbin-1 may be an important molecule in understanding T-LEC function and control of lymphatic metastasis.

1. Introduction

Since lymph node metastasis in cancer is one of the most important prognostic factors, useful diagnostic and therapeutic methods for such pathology are highly desirable [1,2]. Blood vessel formation, or angiogenesis in tumors, is well known to be an essential contributor to tumor metastasis [3–5]. Various approaches have been reported in the study of tumor blood vessels, such as construction of *in vitro* tumor vascular endothelial cell models using conditioned medium (CM) from cancer cells [6–8]. In these studies, proteins highly expressed in tumor vascular endothelial cells have been identified (e.g. vascular endothelial growth factor receptor 2 (VEGFR2), tumor endothelial marker 7 (TEM7) and ROBO4) [9–13]. Moreover, an antibody drug to VEGF has already been used in the clinic [14,15]. As well as tumor blood vessels, lymphangiogenesis in tumors is also considered to be an important factor in lymphatic metastasis. However, the study of tumor lymphatic vessels is at an early stage. One of the reasons is that primary culture of LECs has only been established recently [16,17]. It has been reported that lymphatic vessels or lymphatic endothelial cells in tumors (T-LECs) have more invasive and neogenetic characteristics, which are distinct from those in normal tissues [18,19]. The difference in these characteristics suggests that molecules specific for tumor lymphatic vessels may be expressed. However, little is known about the molecular biology of tumor lymphatic vessels, in contrast to the situation with tumor vascular vessels [3–5]. Recently, proteome analysis has been undertaken, as one approach to the elucidation of events at molecular level, because proteins are the molecules which directly determine vital functions [20,21]. Consequently we focused on tumor lymphatic vessels and using proteome analysis, sought to identify tumor lymphatic endothelial cell (T-LECs)-related proteins to elucidate molecular mechanisms, with a view to development of molecular targeting therapeutic approaches.

In advancing the study according to this strategy, we had two problems. Firstly, it is not possible to collect sufficient amounts of sample, i.e. it is very difficult to isolate LECs from tumor tissues *in vivo* and obtain sufficient amounts for proteome analysis. Therefore, we firstly constructed a T-LEC model *in vitro* using CM from metastatic cancer cells. Because such CM includes various liquid factors produced from metastatic cancer cells, we considered it would appropriately mimic the environment in metastatic tumor tissues. The second problem relates to validation of identified proteins. Because many candidate proteins that are high- or

* Corresponding author at: Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Fax: +81 72 641 9817.
E-mail address: tsunoda@nibio.go.jp (S.-i. Tsunoda).
[1] These authors contributed equally to this work.

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low-expressed in diseased tissues are found by such a procedure, it is important to validate and focus on the appropriate proteins. Therefore we used a tissue microarray approach, employing a large number of normal tissues and tumor tissues and analyzing the relationship between protein expression and clinical information.

In this study, we searched for T-LEC-related proteins by two-dimensional differential in-gel electrophoresis (2D-DIGE) analysis using the T-LEC model, and validated the identified proteins as T-LEC-related by tissue microarray analysis.

2. Materials and methods

2.1. Cell lines

Primary normal human lung lymphatic microvascular endothelial cells (LECs) were purchased from Lonza Inc., (product name; HMVEC-LLy). LECs were confirmed to express the lymphatic markers CD31 and podoplanin in at least 95% of cells by flow cytometry. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 using an EGM-2-MV BulletKit (Lonza) and used in experiments within three passages after purchase. The human lung cancer cells line, RERF-LC-KJ, which is known to have high metastatic potential to lymphnode in immunodeficient mice [22], was purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank and cultured in RPMI1640 supplemented with 10% FCS. Primary normal human bronchial epithelial (NHBE) cells were purchased from Lonza and were maintained using a BEGM BulletKit (Lonza).

2.2. Tumor and normal tissue lymphatic endothelial cell models

Conditioned medium (CM) was prepared from the supernatant of 48 h cultures of RERF-LC-KJ and NHBE cells. The collected supernatants were subsequently filtered (0.2 μm pores) to remove cellular debris. LECs were cultured in a medium containing 10% FCS and 50% tumor cells or NHBE cell CM for 48 h and used as models of lymphatic endothelial cells in tumor (T-LECs) and normal tissue (N-LECs), respectively.

2.3. In vitro tube formation assay

Twentyfour-well culture plates were coated with 300 μl of Matrigel basement membrane matrix (BD Bioscience) per well according to the manufacturer's instructions. LEC suspensions (250 μl), each containing 10% FCS and 50% of either CM, were seeded on the Matrigel-coated wells (3×10^4 cells/well). After incubation at 37 °C for 6 h, cells were stained with Calcein-AM solution (Dojindo Laboratories Co.) and images captured with a fluorescence microscope (Power IX81, Olympus). Tube formation of LECs was quantified as tube length per set of eight randomly selected fields per group using image analysis software (MetaXpress, Molecular Devices, Inc.,).

2.4. In vitro invasion assay

LECs were pre-cultured for 24 h in serum-free EBM-2 medium (Takara Bio). The membrane of 96-well chamber plates was coated with 50 μl of basement membrane extract (Cultrex) and dried overnight at 37 °C. LECs (5×10^4 cells per well) were added to the upper chambers and 150 μl of medium containing 10% FCS and 50% of either CM were added to the lower chambers. After incubation for 48 h at 37 °C, the non-invasive cells on the upper side of the membranes were removed by scrubbing. The invasive cells in the lower chambers were quantified by staining with Calcein-AM solution and a fluorescence microplate reader.

2.5. 2D-DIGE analysis

Proteome analysis was performed by 2D-DIGE and mass spectrometry. LECs cultured in CM of RERF-LC-KJ cells and NHBE cells for 48 h were solubilized with 7 M urea, 2 M thiourea, 4% CHAPS and 10 mM Tris–HCl (pH 8.5). The lysates from each LEC type were purified using a 2D-Clean up kit (GE Healthcare Biosciences) and labeled with Cy3 or Cy5 protein-labeling dye (GE Healthcare Biosciences) at a ratio of 50 μg protein: 400 pmol dye according to the manufacturer's instructions. For the first isoelectric focusing separation by 2D-electrophoresis, 50 μg of each labeled sample was mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% Pharmalyte (GE Healthcare Biosciences)) and applied to a 24-cm immobilized pH gradient gel strip (IPG-strip pH 4–7 NL, GE Healthcare Biosciences). The samples for the spot-picking gel were prepared without Cy-dye labelling. For the second dimension separation, the IPG-strips were applied to SDS–PAGE gels (10% polyacrylamide and 2.7% *N,N′*-diallyltartardiamide). After electrophoresis, the gels were scanned with a fluoroimager (Typhoon Trio, GE Healthcare Biosciences). The spot-picking gel was also scanned after staining with Flamingo solution (Bio-Rad). Quantitative analysis of protein spots was carried out with Decyder-DIA software (GE Healthcare Biosciences). Protein spots of differential expression were picked using an Ettan Spot Picker (GE Healthcare Biosciences).

2.6. In-gel tryptic digestion

The picked gel pieces were twice destained with 50% acetonitrile/50 mM NH_4HCO_3 for 20 min, dehydrated with 75% acetonitrile for 20 min, and then dried using a centrifugal concentrator. Next, 5 μl of 20 μl/ml mass spectrometry grade trypsin solution (Promega) was added to each gel piece and incubated for 16 h at 37 °C. To extract the resulting peptides from the gel pieces, they were treated with a series of acetonitrile/trifluoroacetic acid (TFA) solution. First, 50 μl of 50% (v/v) acetonitrile in 1% (v/v) aqueous TFA was added to the gel pieces, which were then sonicated for 5 min. Next, the solution was collected and 80% (v/v) acetonitrile in 0.2% TFA added. 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_{18} pipette tips (Millipore). 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. Protein identification by mass spectrometry

The tryptic digests (0.6 μl) were mixed with 0.6 μl α-cyano-4-hydroxy-trans-cinnamic acid saturated in a 0.1% TFA and acetonitrile (1:1 vol/vol). Each mixture was deposited onto the well of a target plate and then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (AutoflexII, Bruker Daltonics). The Mascot search engine (Matrixscience) was initially used to query the entire theoretical tryptic peptide as well as the SwissProt protein sequence database.

2.8. Tissue microarray analysis

Human lung cancer and normal tissue microarrays (Biomax) were deparaffinated in xylene and rehydrated in a graded series of ethanol concentrations. 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_2O_2 in TBS for 5 min followed by washing twice in TBS. After blocking with 5% BSA solution, the slides were incubated for 60 min with the following antibodies: anti-podoplanin (Dako), anti- HYOU-1 (Abnova), anti-hnRNPK (Abcam), anti-GRIM-12 (Affinity BioReagents) anti-vimentin (Abcam) and anti-reticulocalbin-1 (Novus Biologicals). After washing with the wash buffer (Dako), each array was stained with Envision + Dual Link (DAKO) and 3, 3′-diaminobenzidine. After development, arrays were lightly counterstained with Mayer's hematoxylin, and mounted with resinous mounting medium. All procedures were performed using AutoStainer (Dako).

2.9. Tissue microarray scoring

The optimized staining condition for lung tumor microarray was determined based on the coexistence of both positive and negative cells in the same tissue sample. Stain was considered positive when reaction products were localized in the expected cellular component. The criteria for evaluation were 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 stain (intensity score) was scored as 0 (no signal), 1 (weak), 2 (moderate), and 3 (marked). The sum of distribution score and intensity score was then calculated as 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.

3. Results

3.1. Evaluation of T-LEC model

In order to confirm that the *in vitro* tumor lymphatic vessel model reflected properties of *in vivo* tumor lymphatic vessels, tube formation and invasiveness were examined. The tube formation assay showed that the tube length of LECs in the RERF-LC-KJ CM group was significantly greater than that in the normal cell CM group (Fig. 1A). The invasion assay showed that the number of invasive LECs in the RERF-LC-KJ CM group was also significantly greater than that in the normal CM group (Fig. 1B). No difference between groups was observed in cell proliferation (data not shown). These data suggested that the *in vitro* T-LEC model using CM reflected properties of *in vivo* tumor lymphatic vessels, at least as regards enhanced lymphangiogenesis.

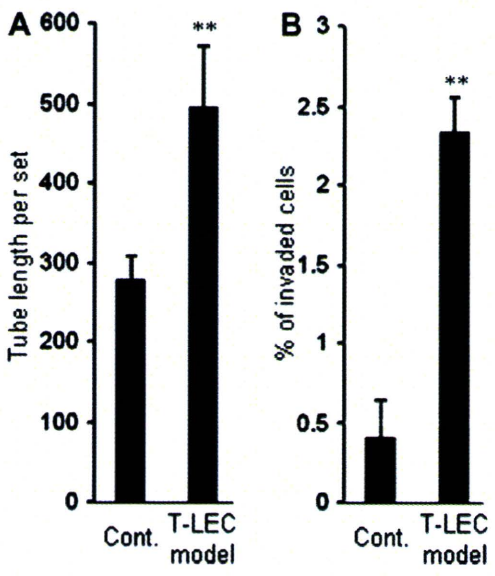

Fig. 1. Enhancement of lymphangiogenesis by treatment with CM of metastatic lung cancer cells. Effect on lymphangiogenesis of treatment with CM from the metastatic lung cancer RERF-LC-KJ cell line was assessed by (A) tube formation assay and (B) invasion assay. Error bars indicate the mean ± S.D. of triplicate assays (**$P < 0.01$).

3.2. Differential proteome analysis in T-LEC and N-LEC models

In order to search for T-LEC-related proteins, we performed differential proteome analysis of the T-LEC and N-LEC models (Fig. 2). By quantitative image analysis, protein spots representing >1.5-fold alteration in expression were found and identified by mass spectrometry (Table 1).

3.3. Validation of the candidate proteins using tissue microarrays

In order to validate the identified proteins as T-LEC-related proteins, we analyzed their expression profile using the lymphatic vessel tissue microarray in lung tumor and normal lung tissues. Results showed that heterogeneous nuclear ribonucleoprotein K (hnRNPK), gene associated with retinoid interferon-induced mortality 12 (GRIM-12) and vimentin were expressed both in T-LECs and N-LECs. In contrast, hypoxia up-regulated protein 1 (HYOU1) was expressed in neither T-LECs nor N-LECs (data not shown). Interestingly, reticulocalbin-1 was specifically expressed in T-LECs, while podoplanin, a recognized lymphatic vessel marker, was expressed in all T-LECs and N-LECs (Fig. 3). Moreover, the expression profiles of these molecules in lung cancer tissues and normal lung tissues were analyzed (Table 2). The result from tissue microarray analysis showed that GRIM-12 and vimentin were expressed in normal lung and tumor tissues. On the other hand, HYOU1 was specifically expressed in approximately 40% of lung cancer patients, while hnRNPK and reticulocalbin-1 were expressed in approximately 70% of such patients.

4. Discussion

This paper is the first report demonstrating differential proteome analysis of T-LEC and N-LEC models using CM of metastatic lung cancer cells and normal cells. We first found that the T-LEC model, obtained from LECs cultured in CM of metastatic cancer

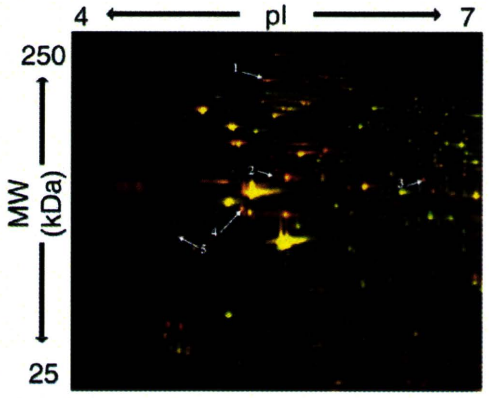

Fig. 2. 2D-DIGE image of fluorescently labeled LEC proteins cultured in CM of metastatic lung cancer cells and normal bronchial epithelial cells. Proteins prepared from LEC cultured in CM from the metastatic lung cancer RERF-LC-KJ cell line, and from normal bronchial epithelial cells (NHBE), were labeled with Cy3 and Cy5, and used for 2D-DIGE analysis. The spots indicated by arrows show the proteins identified by MALDI-TOF/MS.

Table 1
Identification of differentially-expressed proteins in T-LEC model by MALDI-TOF/MS.

Spot	Protein name	Accession No.	MW (kDa)	pI	Expression ratio (fold) [Cancer CM/Normal CM]
#1	HYOU1	Q9Y4L1	111	5.2	1.7
#2	hnRNPK	P61978	48	5.5	2.0
#3	GRIM-12	Q16881	55	6.4	1.8
#4	Vimentin	P08670	54	5.1	1.9
#5	Reticulocalbin-1	Q15293	39	4.9	1.9

Table 2
Positive rate of identified proteins in lung cancer and normal lung tissues.

Protein name	Positive rate of identified proteins			
	Normal lung tissues		Lung cancer tissues	
Podoplanin	11/29 cases	(38%)	70/221 cases	(32%)
HYOU1	0/29 cases	(0%)	87/221 cases	(39%)
hnRNPK	0/29 cases	(0%)	153/221 cases	(69%)
GRIM-12	3/29 cases	(10%)	114/221 cases	(52%)
Vimentin	15/29 cases	(52%)	90/221 cases	(41%)
Reticulocalbin-1	0/29 cases	(0%)	158/221 cases	(72%)

cells, was a useful example to use in searching for candidate tumor lymphatic endothelial cell markers, since it showed more invasive and neogenetic characteristics than that of normal cells in *in vitro* experiments. Since induction of vascular angiogenesis in tumors serves to provide nutrients, lymphangiogenesis induction in tumors is considered to facilitate removal of waste material. Thelen has reported that higher levels of lymphatic vessel density were related to a higher degree of metastasis, by statistical analysis of clinical specimens [23]. This report suggests that lymphatic endothelial cells in metastatic tumors are probably activated and induce their migration *via* soluble factors obtained from the tumor cells. The T-LEC model used in our experiments is considered to be activated by CM from metastatic tumor cells.

Using the T-LEC and N-LEC models, we performed differential proteome analysis to search for marker proteins expressed on T-LECs and identified a candidate protein reticulocalbin-1. Although lymphatic vessel endothelial hyaluronic acid receptor-1 (Lyve-1), podoplanin and vascular endothelial growth factor receptor-3 (VEGFR3) are known as lymphatic markers, these markers do not show specificity for T-LECs [24–26]. Interestingly, reticulocalbin-1 is shown to be expressed in T-LECs, but not N-LECs by TMA analysis. Reticulocalbin-1 is a member of the family of Ca^{2+}-binding proteins localized in the endoplasmic reticulum and is suggested to function in the secretory pathway of cells [27,28]. It is also reported that malignancy of hepatocellular carcinoma cell lines is increased by over-expression of reticulocalbin-1 [29]. Furthermore, a highly invasive mammary cancer cell line has been shown to express reticulocalbin-1, while a poorly invasive cancer cell line did not [30]. Thus, reticulocalbin-1 may be involved in invasion of lymphatic vessels into tumor tissues.

Since the identified proteins were validated using TMAs from many clinical specimens of lung cancer cases and normal tissues, we were able to identify the several candidates specifically expressed in lung cancer cells, namely HYOU1, hnRNPK and reticulocalbin-1. HYOU1 is induced by hypoxia and has a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation [31]. hnRNPK is a conserved RNA-binding protein that is involved in multiple processes of gene expression, including chromatin remodeling, transcription, and mRNA splicing, translation, and stability [32,33]. Recently HYOU1 expression in breast and colorectal cancer, and hnRNPK expression in breast cancer have been reported [34–36]. However, the relevance of these proteins to lung cancer has not been reported. Our data suggest that these proteins could be diagnostic and therapeutic targets in lung cancer. Furthermore, reticulocalbin-1 was highly expressed in lung tumor tissues compared to normal lung tissues, as well as being highly expressed in T-LECs compared to N-LECs. In addition, reticulocalbin-1 is reported to be expressed on cell membranes of some cancer cells [37]. Consequently, it could be a useful target for antibody therapy in cancer metastasis, at least in terms of expression profile. Further work is required to reveal the functions of these proteins in lung cancer.

In conclusion, we have identified reticulocalbin-1 as candidate T-LEC-related protein in lung tumors for the first time. Furthermore we have shown that HYOU1 and hnRNPK as highly expressed in lung tumors, by differential proteome analysis of the T-LEC model using CM. We hope that identified T-LEC-related proteins will contribute to advances in molecular biology and the development of diagnostic and therapeutic methods.

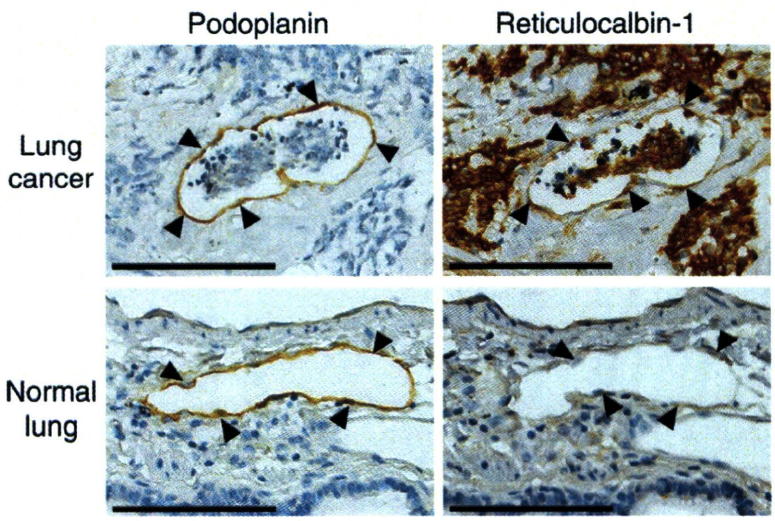

Fig. 3. Immunohistochemical staining of lymphatic vessels in human lung cancer and normal lung tissues using anti-podoplanin and anti-reticulocalbin-1 antibodies. Lymphatic vessels in human lung cancer and normal lung tissues were immunostained with anti-podoplanin and anti-reticulocalbin-1 antibodies and counter-stained by hematoxylin. The arrowheads indicate lymphatic endothelial cells. Scale bar is 100 μm.

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