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厚生労働科学研究費補助金

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 24 年度　　総括研究報告書

平成 22-24 年度　総合研究報告書

研究代表者　長野一也

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総括研究報告書

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

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、分子病態の解明、及び有用な診断法・治療法の開発に資する知見を収集することを目的とする。

上記を達成するため、昨年度までに、①正常中皮細胞と悪性中皮腫細胞、②シスプラチン(CDDP)高感受性細胞と低感受性細胞におけるプロテオーム解析により、共通して発現変動していた3種類の悪性中皮腫関連バイオマーカー候補蛋白質を同定した。その中で、発現変動率の大きかったAnnexin A4(ANXA4)に対し、独自に作製したファージ抗体を用いて臨床検体を免疫染色した結果、正常中皮組織に比べ、悪性中皮腫組織で高率に発現しており、悪性中皮腫に対して特異性の高い蛋白質であることが示された。また、各種悪性中皮腫細胞のCDDPに対する感受性とANXA4の発現量が、負に相関する傾向が認められ、CDDPの抵抗性に関連する可能性が示された。

以上の成果をふまえ、本年度はANXA4のCDDP抵抗性への関与を明らかにするため、遺伝子工学的にANXA4の発現量を変化させた結果、ANXA4のノックダウンによりCDDPの感受性は有意に亢進し、逆に強制発現させることで、その感受性は有意に低下した。以上より、ANXA4は、悪性中皮腫細胞におけるCDDP抵抗性に関わる機能を有していることが明らかとなった。

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推進されている。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。本観点から研究代表者らは、プロテオーム解析から直接同定・回収される微量な候補蛋白質を、吸着力に優れるニトロセルロース膜に効率よく固相化することで、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マイクロアレイを免疫染色し、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解析から、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、創薬バイオマーカー蛋白質を効率よく探索可能な「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、本疾患の分子病態の解明や有用な診断法・治療法の開発に資する知見を収集すると共に、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。その一方で、本疾患に対する的確な診断・有効な治療法はなく、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、昨年度までの2年間で研究を進めてきた結果、正常中皮組織に比べ、悪性中皮腫組織で高率に発現し、各種悪性中皮腫細胞株のCDDP抵抗性と発現が負の相関傾向をもつAnnexin A4(ANXA4)を世界に先駆けて見出してきた。そこで、本年度は、ANXA4のCDDP抵抗性への関与を明らかにするため、遺伝子工学的にANXA4の発現量を変化させたところ、ANXA4のノックダウンによりCDDPの感受性は有意に亢進し、逆に強制発現させることで、その感受性は有意に低下した。

以上より、ANXA4は、悪性中皮腫細胞におけるCDDP抵抗性に関わる機能を有していることが明らかとなったので、これらの研究成果を報告する。

B. 研究方法

B-1. 細胞培養

悪性中皮腫細胞株（H28、H2052）はATCCより購入したものを用いた。培養には、終濃度10%になるようにFCSを添加したRPMI1640培地を用い、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。

B-2. CDDPによる細胞傷害性試験

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CDDPを添加し、24時間後の細胞傷害性をWST-8 assayにより評価した。

B-3. ANXA4 のノックダウン

ANXA4 に対する siRNA（配列：AAGGATATCACAGAAGGATAT)は、Qiagen より購入した。Hyperfect regent (Qiagen)を用いて、ANXA4 siRNA を ANXA4 が発現する CDDP 抵抗性の H28 細胞にトランスフェクションし、B-2.に従って、CDDP に対する感受性変化を評価した。

B-4. ANXA4 の強制発現

ANXA4 遺伝子を pcDNA3.1 ベクターにクローニングしたプラスミドを、FuGENE HD transfection reagent (Roche)により、H2052 細胞(ANXA4 が低発現で CDDP 感受性株)にトランスフェクションし、B-2.に従って、CDDP に対する感受性変化を評価した。尚、ANXA4-pcDNA3.1 は、(独)医薬基盤研究所免疫シグナルプロジェクト　仲　哲治先生より供与いただいた。

B-5. Western Bloting による ANXA4 発現解析

遺伝子工学的に処理された悪性中皮腫細胞株（H28、H2052）を細胞溶解液により可溶化し、2倍濃度のLaemmli Sample Bufferを等量混合した。終濃度 5%となるように 2-mercaptoethanol を添加後、95 ℃で 5 分処理した。各試料を、SDS-PAGE用ゲルに添加し、電気泳動を行い、蛋白質を分離した。その後、ゲルを PVDF 膜に転写し、10% Block Ace にて室温、1 時間インキュベーションすることでブロッキングした。TBS で 1 回洗浄後、mouse anti-human ANXA4 antibody (Abnova : 1D3)を添加し、緩やかに振とうさせながら室温で 1 時間反応させた。TBST（0.05% Tween 20 を含む TBS）にて 3 回洗浄した後、HRP/anti-mouse IgG monoclonal antibody を添加し、振とうさせながら室温で 1 時間反応させた。TBST で 3 回洗浄後、メンブレンを ECL plus Western Blotting Detection System で処理し、LAS-3000 により検出した。

C. 研究結果

C-1. ANXA4のノックダウンによるCDDP感受性の亢進

昨年度までに研究代表者は、①正常中皮細胞と悪性中皮腫細胞、②シスプラチン(CDDP)高感受性細胞と低感受性細胞におけるプロテオーム解析から、ANXA4を見いだし、本蛋白質が(1)正常中皮組織に比べ、悪性中皮腫組織で高率に発現しており、悪性中皮腫に対して特異性の高い蛋白質であること、(2)各種悪性中皮腫細胞のCDDPに対する感受性とANXA4の発現量が、負に相関する傾向が認められ、CDDPの抵抗性に関連する可能性を示してきた。そこで本年度は、ANXA4のCDDP抵抗性への関与を明らかにするため、まず、CDDP抵抗性株であるH28 中のANXA4の発現をノックダウンさせ、CDDPの感受性変化を評価した。

ANXA4に対するsiRNAをH28にトランスフェクションし、ANXA4の発現がノックダウンされていることをWestern Blotにより確認した(Fig. 1 (b))。そのうえで、各濃度のCDDPを添加し、その感受性をNon-treat群/Control siRNAトランスフェクション群と比較解析した。その結果、対照群に比較して、ANXA4 siRNAトランスフェクション群で有意に感受性が亢進することが明らかとなった(Fig. 1 (a))。

尚、siRNAのトランスフェクションによる細胞傷害がないことを確認している。

C-2. ANXA4の強制発現によるCDDP感受性の低下

ANXA4のCDDP抵抗性への関与を確かめるため、C-1.とは逆に、ANXA4低発現でCDDP感受性の高いH2052にANXA4を強制発現させて、CDDP感受性変化を検討した。ANXA4の蛋白質発現を確認したうえで(Fig. 2 (b))、CDDPに対する細胞傷害性を比較解析した結果、対照群と比較して有意にCDDPに対する感受性が低下した(Fig. 2 (a))。

以上から、ANXA4は、悪性中皮腫細胞におけるCDDPの抵抗性に関わる機能を有していることが示唆された。

今後、実際の臨床検体を用いて、ANXA4の発現プロファイルとCDDP感受性の相関について、後ろ向き/前向きに解析する必要はあるものの、本知見は、悪性中皮腫患者のCDDPの効果を事前に予測し、治療薬選択のためのCDDP抵抗性マーカーになりうることを初めて明らかにした。また、臨床応用に向けては、CDDP抵抗性のメカニズムを明らかにする必要が考えられる。ANXA4は、ANXA1～A13のサブタイプが知られているAnnexin Aファミリーの1つであり、細胞膜の調節因子として、恒常性維持に関与していることが知られている。したがって、膜透過性の向上やエキソサイトーシスなどの機構に着目して、CDDP抵抗性メカニズムを明らかにしていきたいと考えている。さらに、本機構のCDDP特異性(CDDPにのみ

認められる機構なのか、もしくは他の白金製剤や他の抗がん剤にも共通しているのか)や、悪性中皮腫での特異性(悪性中皮腫のみで認められるのか、もしくは他のがんにおいても認められる現象なのか)など、その適用範囲を詰めていく必要も考えられる。このように実用化に向けては数多くの解析が必要であるものの、世界で初めて見いだした本知見が、予後不良な悪性中皮腫の克服の一助になることや、厚生労働行政に貢献することを切に祈念している。

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。

- CDDP抵抗性の悪性中皮腫細胞株のANXA4の発現をノックダウンさせることより、有意にその感受性は亢進した。
- ANXA4低発現でCDDPへの感受性が高い悪性中皮腫細胞株にANXA4を強制発現させることにより、有意にその感受性は低下した。
- 上記の知見から、ANXA4は、悪性中皮腫細胞におけるCDDPの抵抗性に関わる機能を有していることが示唆された。

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

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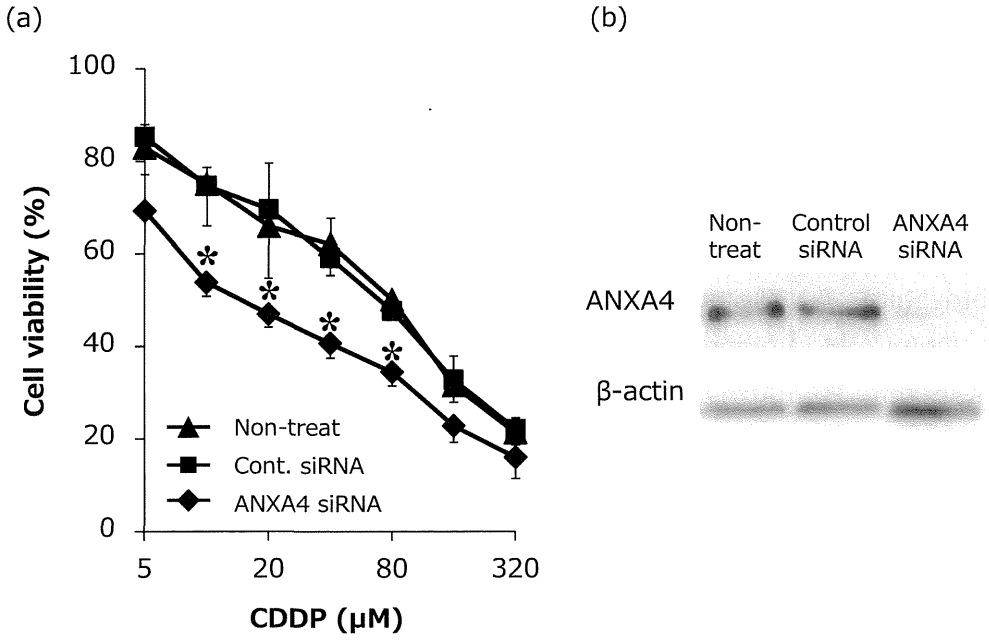

Fig. 1 The effect of ANXA4 gene knockdown on CDDP susceptibility in malignant mesothelioma cells.

Transfection of ANXA4 siRNA into H28 malignant mesothelioma cells confers resistance to CDDP. Cell viability after 36 h treatment of ANXA4 siRNA with different concentrations of CDDP was evaluated by WST-8 assay (a). Expression of ANXA4 was analyzed by western blot analysis (b). Data are shown as means and standard deviations (n=4). *P < 0.05 (Control siRNA-treated group vs ANXA4 siRNA-treated group)

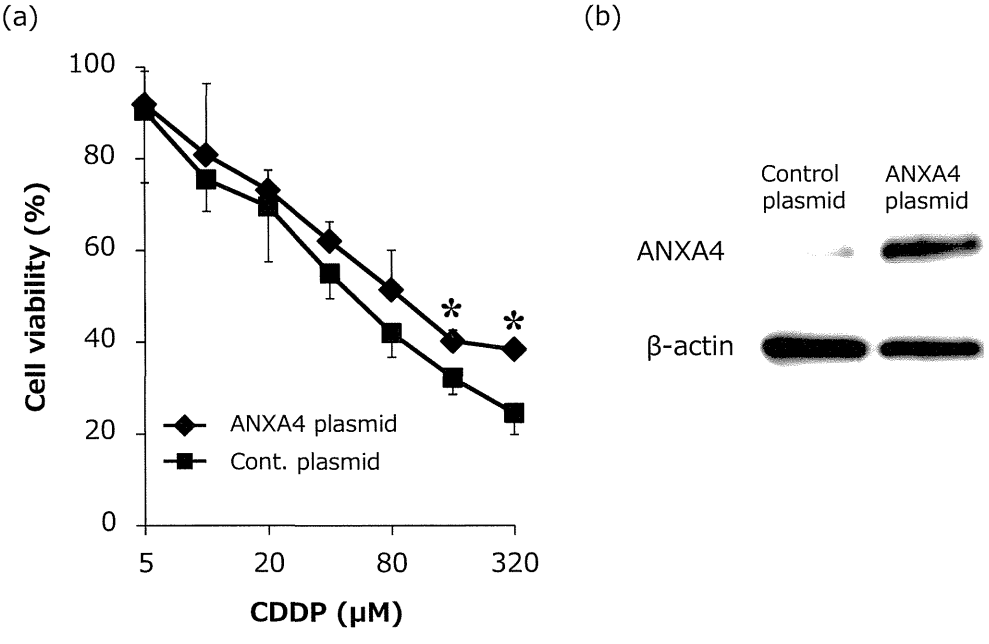

Fig. 2 The effect of ANXA4 gene overexpression on CDDP susceptibility in malignant mesothelioma cells.

Transfection of ANXA4 siRNA into H2052 malignant mesothelioma cells confers resistance to CDDP. Cell viability after 24 h treatment of ANXA4 plasmid with different concentrations of CDDP was evaluated by WST-8 assay (a). Expression of ANXA4 was analyzed by western blot analysis (b). Data are shown as means and standard deviations (n=4). *P < 0.05 (Control plasmid-treated group vs ANXA4 plasmid-treated group)

研究成果の刊行に関する一覧表

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Takano M., Yamashita T., **Nagano K.**, Otani M., Maekura K., Kamada H., Tsunoda S., Tsutsumi Y., Tomiyama T., Mori H., Matsuura K., Matsuyama S.	Proteomic analysis of the hippocampus in Alzheimer's disease model mice by using two-dimensional fluorescence difference in gel electrophoresis.	***Neuroscience Letters***	534	85-89	2013
Yamashita T., ***Nagano K.**, Kanasaki S., Maeda Y., Furuya T., Inoue M., Nabeshi H., Yoshikawa T., Yoshioka Y., Itoh N., Abe Y., Kamada H., Tsutsumi Y., Tsunoda S.	Annexin A4 is a possible biomarker for cisplatin susceptibility of malignant mesothelioma cells.	***Biochem. Biophys. Res. Commun.***	421 (1)	140-144	2012
Yamashita T., Okamura T., ***Nagano K.**, Imai S., Abe Y., Nabeshi H., Yoshikawa T., Yoshioka Y., Kamada H., Tsutsumi Y., Tsunoda S.	Rho GDP-dissociation inhibitor alpha is associated with cancer metastasis in colon and prostate cancer.	***Pharmazie***	67 (3)	253-255	2012

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Proteomic analysis of the hippocampus in Alzheimer's disease model mice by using two-dimensional fluorescence difference in gel electrophoresis

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HIGHLIGHTS

- We perform the proteome for $APP_{E693\Delta}$-transgenic mice. Methods are two-dimensional fluorescence difference in gel electrophoresis and mass spectrometry techniques. The expression of 14 proteins are changed in the brain. Aβ oligomers contribute to the expression of proteins.

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ABSTRACT

We previously identified the E693Δ mutation in amyloid precursor protein (APP) in patients with Alzheimer's disease (AD) and then generated APP-transgenic mice expressing this mutation. As these mice possessed abundant Aβ oligomers from 8 months of age but no amyloid plaques even at 24 months of age, they are a good model to study pathological effects of amyloid β (Aβ) oligomers. The two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) technology, using a mixed-sample internal standard, is now recognized as an accurate method to determine and quantify proteins. In this study, we examined the proteins for which levels were altered in the hippocampus of 12-month-old $APP_{E693\Delta}$-transgenic mice using 2D-DIGE and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Fourteen proteins were significantly changed in the hippocampus of $APP_{E693\Delta}$-transgenic mice. Actin cytoplasmic 1 (β-actin), heat shock cognate 71 kDa, γ-enolase, ATP synthase subunit β, tubulin β-2A chain, clathrin light chain B (clathrin) and dynamin-1 were increased. Heat shock-related 70 kDa protein 2, neurofilament light polypeptide (NFL), stress-induced-phosphoprotein 2, 60 kDa heat shock protein (HSP60), α-internexin, protein kinase C and casein kinase substrate in neurons protein 1 (Pacsin 1), α-enolase and β-actin were decreased. Western blotting also validated the changed levels of HSP60, NFL, clathrin and Pacsin 1 in $APP_{E693\Delta}$-transgenic mice. The identified proteins could be classified as cytoskeleton, chaperons, neurotransmission, energy supply and signal transduction. Thus, proteomics by 2D-DIGE and LC–MS/MS has provided knowledge of the levels of proteins in the early stages of AD brain.

1. Introduction

AD is neuropathologically characterized by abnormal accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles throughout cortical and limbic regions. Although the current amyloid cascade hypothesis [6] and tau hypothesis [15] provide frameworks for studying AD pathogenesis. Recently, diverse lines of evidence suggest that Aβ peptides play more important roles in AD pathogenesis [13,16,20]. Especially, soluble oligomers of Aβ could be a cause of synaptic and cognitive dysfunction in the early stages of AD. To address the relationship between Aβ oligomers and pathological features of AD, we generated APP transgenic mice expressing the E693Δ mutation, which enhanced Aβ oligomerization without fibrillization [25]. It might provide a clue for elucidating AD pathology caused by Aβ oligomers to analyze the $APP_{E693\Delta}$-transgenic mice.

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One of the most utilized approaches in proteomics to quantify and identify proteins is two dimensional gel electrophoresis (2DE) and mass spectrometry (MS) [5]. Proteomic approaches were most widely based on methods using differential expression on 2D-PAGE gels, or more recently 2D chromatography, followed by mass spectrometry protein identification. Compared to these conventional analyses, 2D-DIGE has higher reproducibility and sensitivity because of its internal standard design which minimizes gel-to-gel variation, improves spot matching, reduces number of gels needed, and permits quantitative analysis of small sample amounts.

In this study, we studied the altered expression of proteins in the hippocampus of $APP_{E693\Delta}$-transgenic mice using 2D-DIGE and LC–MS/MS approach. This approach revealed that the levels of at least 14 proteins were altered in the hippocampus of 12-month-old $APP_{E693\Delta}$-transgenic mice. These findings suggest that Aβ oligomers might cause synaptic and cognitive dysfunction by affecting the expression of these proteins in the hippocampus.

2. Experimental procedures

2.1. Materials

Sodium dodecyl sulfate, urea, thiourea, CHAPS, dithiothreitol, iodoacetamide, bromophenol blue, and RNase A and DNase I for SDS-PAGE or 2DE were all obtained from Wako Pure Chemical Industries (Osaka, Japan). Source information for all other assay reagents and materials are incorporated into their respective assay methods described below.

2.2. Animal subjects

Transgenic mice expressing human APP_{695} with the APP E693Δ mutation under the mouse prion promoter were used [25]. Heterozygous human $APP_{E693\Delta}$-transgenic mice and age-matched non-transgenic littermates were sacrificed at 12 months of age, and their hippocampi were isolated on an ice-cold plate. Animal care and handling were performed strictly in accordance with the Guidelines for Animal Experimentation at Kobe Gakuin University and Himeji Dokkyo University. Every effort was made to minimize the number of animals used and their suffering.

2.3. Protein labeling with CyDyes

Equal amounts of total protein from 4 hippocampi of $APP_{E693\Delta}$-transgenic mice or age-matched non-transgenic littermates were separately pooled. Protein samples were labeled with CyDyes (GE Healthcare, Piscataway, NJ), as per manufacturer's instructions. In brief, 50 μg of total protein from each sample was mixed in a tube and labeled with Cy2 minimal dye, and 50 μg protein taken from the mix was used as an internal standard on each gel for the three subsequent 2DE and image analysis. In parallel, 50 μg protein from each sample was labeled with either Cy3 or Cy5, and the dyes scrambled within each group to avoid possible dye bias. As a result, one replicate was Cy3 labeled proteins and another replicate was Cy5 labeled proteins. Two replicates (Cy3 and Cy5 labeled samples) were mixed, divided and applied each three independent gels. The sample volumes were adjusted to 18 μL with labeling buffer (7M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris), followed by addition of 1 μL dye (working solution) to each individual sample. The samples were left on ice for 30 min in the dark, followed by adding 1 μL of 10 mmol/L lysine to stop the reaction.

2.4. 2D electrophoresis and image analysis

One sample from each of the CyDye groups was mixed together and adjusted to final concentrations of 1% DTT, 1% IPG buffer at a total volume of 350 μL with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) and was used to 24 cm pH 4–7 IPG strips (non-linear; GE Healthcare, Piscataway, NJ) overnight. First dimension isoelectric focusing (IEF) was carried out with IPGphor II (GE Healthcare, Piscataway, NJ). Second dimension SDS-PAGE was performed by mounting the IPG strips onto 20 × 26 cm 12.5% DIGE gels (GE Healthcare, Piscataway, NJ) using Ettan DALT six Large Electrophoresis System (GE Healthcare, Piscataway, NJ) and running the gels at 16 mA/gel for the initial hour and 25 mA/gel at 25 °C constantly until bromophenol blue reached the bottom of the gel. The lysates were labeled at the ratio of 50 μg proteins: 400 pmol Cy3 or Cy5 protein-labeling dye (GE HealthcareBiosciences) in dimethylformamide according to the manufacturer's protocol.

In summary, three analytical gels were completed in total, running 25 μg of pooled reference sample labeled with Cy2, along with two samples (25 μg each), one labeled with Cy3 and the other labeled with Cy5. Gels selected for picking were stained with Deep purple (GE Healthcare, Piscataway, NJ). Approximately 1100 spots were matched across all three analytical gels. The analytical gel was picked using an automated robotic system, Ettan Spot picker (GE Healthcare, Piscataway, NJ). The pick list was created based on the Deep purple image. 2 mm gel plugs were picked, washed, reduced and alkylated, and then digested with trypsin, and the resulting peptides were extracted. Gel trypsinization was performed as previously described [24].

2.5. LC/MS/MS identification

Trypsinized peptides were analyzed by nano LC/MS/MS on a ThermoFisher LTQ Orbitrap XL. In brief, 30 mL of hydrolysate was loaded onto a 5 mm 675 mm ID C12 (Jupiter Proteo, Phenomenex) vented column at a flow-rate of 10 mL/min. Gradient elution was conducted on a 15 cm by 75 mm ID C12 column at 300 nL/min. A 30 min gradient was employed. The mass spectrometer was operated in a data-dependent mode, and the six most abundant ions were selected for MS/MS. Mass spectrometry results were searched using Mascot (www.matrixscience.com). Samples were processed in the Scaffold algorithm using DAT files generated by Mascot. Parameters for LTQ Orbitrap XL data require a minimum of two peptide matches per protein with minimum probabilities of 90% at the protein level.

2.6. Western blotting

Approximately 25 μg of protein from mouse hippocampus was applied to a 12.5% acrylamide gel and SDS-polyacrylamide gel electrophoresis was performed at 17.5 mA/gel for 2 h in second dimension. The gels were transferred onto PVDF membranes (Pall Corporation, Pensacola, FL, USA), in a trans-blot electrophoresis transfer cell (Nihon Eido, Tokyo, Japan). Western blotting was performed by using monoclonal antibodies against β-actin (diluted 1:1000, Cell Signaling, USA) and clathrin (diluted 1:250, Abcam, USA), polyclonal antibodies HSP60, NFL, voltage-dependent anion-selective channel protein 1 (VDAC) (diluted 1:1000, Cell Signaling, USA) and Pacsin 1 (diluted 1:500, Millipore, USA). Peroxidase-conjugated antibody (diluted 1:5000, Abcam, USA) was used as secondary antibody. The reaction was detected by chemiluminescence with ECL reagents (Pierce Biotechnology, USA). A semi quantitative analysis based on optical density was performed by ImageJ software (available at http://www.rsbweb.nih.gov/ij).

3. Results and discussion

The 2D-DIGE gels of the hippocampi from wild type and $APP_{E693\Delta}$-transgenic mice pools were shown as Fig. 1. Two replicates of each pooled sample were run, labeling one replicate with

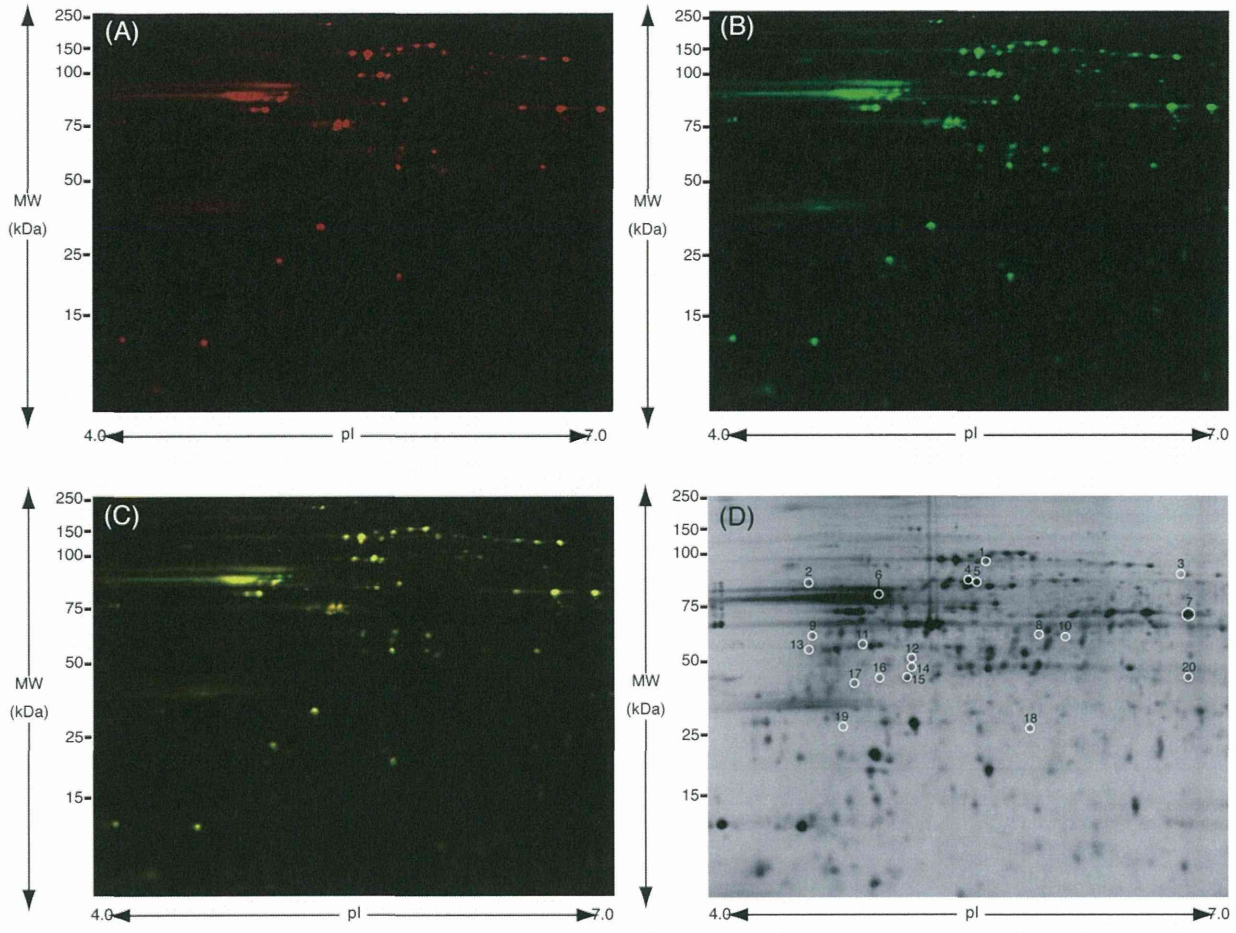

Fig. 1. 2D-DIGE gel image of fluorescence-labeled hippocampal proteins of non-transgenic and $APP_{E693\Delta}$-transgenic mice. (A) Analysis of the proteome of non-transgenic mice hippocampi with Cy3 Dye. (B) $APP_{E693\Delta}$-transgenic mice hippocampi with Cy5 Dye. (C) Merged. (D) Fourteen protein spots identified from non-transgenic and $APP_{E693\Delta}$-transgenic mice hippocampi by LC/MS/MS. Black numbers with white circles indicate proteins that are listed in Table 1.

Cy3 (Fig. 1A) and one replicate with Cy5 (Fig. 1B), resulting in three analytical gels. The 2D-DIGE comparative analysis of the wild type and $APP_{E693\Delta}$-transgenic mice revealed significant 74 spots (Fig. 1C). These spots were investigated by LC–MS/MS (Fig. 1D). Finally, fourteen proteins were identified as shown in Table 1. These proteins are classified into several groups that are involved in cytoskeletal, chaperone, energy metabolic, vesicle transport and signaling proteins (Table 2).

Spot nos. 1, 3 and 4 were identified as heat shock-related 70 kDa protein 2, stress-induced-phosphoprotein 1 and HSP60, respectively. The stress-induced-phosphoprotein 1 is the co-chaperone and thought of the function in regulation of interaction with Hsp70 and Hsp90 [10]. HSP60 is the chaperonin which is implicated in mitochondrial protein import and macromolecular assembly and may facilitate the correct folding of imported proteins [9]. The amounts of heat shock-related 70 kDa protein 2, stress-induced-phosphoprotein 1, and HSP60 were significantly decreased. On the contrary, spot no. 9 which was identified as heat shock cognate 71 kDa protein was significantly increased. This protein is also the chaperone and acts as a repressor of transcriptional activation [8]. Thus, Aβ oligomers might contribute to changing the expression of the chaperons.

Spot nos. 8, 10–12 and 16 were identified as actin, and spot nos. 15 and 17 were identified as tubulin β-2A chain. Actin is one of the major cytoskeletal proteins in neurons, and the dynamics of its assembly are involved in many aspects of cell motility, vesicle transport, and membrane turnover [14]. Actin itself is known to link with Aβ, which enhances the neurotoxicity induced by tau-mediated actin filament formation [4]. The four spots of actin but not no. 12 and those of tubulin were significantly increased. Thus, Aβ oligomers might lead to increasing the amounts of actin and tubulin.

Spot nos. 5 and 2 were identified as α-internexin and NFL, respectively, which are known as neuronal intermediate proteins [2,18]. The amounts of α-internexin and NFL were significantly decreased. Thus, the decreased amounts of NFL and internexin might raise neural dysfunction in the hippocampus of AD.

Spot nos. 7 and 13 were identified as α-enolase. Spot nos. 14 and 19 were identified as γ-enolase and ATP synthase subunit β, respectively. Enolase is a multifunctional protein as glycolytic enzyme, belonging to a novel class of surface proteins [11]. ATP synthase is a key role enzyme that provides energy for the cell to use through the synthesis of ATP [1]. The amount of α-enolase was significantly decreased, but the amounts of γ-enolase and ATP synthase subunit β were significantly increased. Interestingly, the levels of α-enolase and ATP synthase subunit α mitochondrial proteins significantly increased in the hippocampus of J20 Tg mice with amyloid deposition [19]. The amyloid deposit enhanced the expression of energy metabolic proteins [22]. Combined with our findings, both Aβ oligomers and amyloid deposition might play an important role in the change of energy metabolic proteins as α-enolase, γ-enolase and ATP synthase subunit β.

Spot no. 20 was identified as dynamin. Dynamin, a well studied neuron-specific mechanochemical GTPase, pinches off synaptic vesicles, freeing them from the membrane and allowing them to re-enter the synaptic vesicle pool to be refilled for future release

Table 1
Identified proteins from differentially expressed in the hippocampus of$APP_{E693\Delta}$-transgenic mice when compared to non-transgenic littermates.

Spot no.	Protein ID	Fold (APP/WT)	*t*-Test	Accession	Coverage	#Peptides	Predicted MW (kDa)	Calc. pI	Score
1	Heat shock-related 70 kDa protein 2	−1.32	0.040	P14659	26.22	23	69.6	5.67	625.70
2	Neurofilament light polypeptide	−1.48	0.002	P08551	39.96	43	61.5	4.64	1004.84
3	Stress-induced-phosphoprotein 1	−1.44	0.002	Q60864	16.21	9	62.5	6.80	157.49
4	60 kDa heat shock protein	−1.36	0.013	P63038	52.71	71	60.9	6.18	1916.39
5	Alpha-internexin	−1.34	0.023	P46660	42.66	39	55.7	5.27	1119.47
6	Protein kinase C and cascin kinase substrate in neurons protein 1	−1.48	0.023	Q61644	28.34	15	50.5	5.24	356.92
7	Alpha-enolase	−1.32	0.000	P17182	34.33	24	47.1	6.80	474.21
8	Actin, cytoplasmic 1	1.51	0.003	P60709	25.87	14	41.7	5.48	231.79
9	Heat shock cognate 71 kDa protein	1.35	0.015	P63017	12.54	16	70.8	5.52	319.85
10	Actin, cytoplasmic	1.34	0.004	P60709	24.27	13	41.7	5.48	279.37
11	Actin, cytoplasmic 1	1.38	0.022	P60709	15.47	7	41.7	5.48	243.14
12	Actin, cytoplasmic 1	−1.56	0.013	P60709	22.67	12	41.7	5.48	131.57
13	Gamma-enolase	1.33	0.005	P17183	20.05	13	47.3	5.11	237.25
14	ATP synthase subunit beta	1.40	0.047	P56480	23.60	18	56.3	5.34	356.19
15	Tubulin beta-2A chain	1.31	0.021	Q13885	14.83	13	49.9	4.89	313.07
16	Actin, cytoplasmic 1	1.47	0.002	P60709	6.93	3	41.7	5.48	97.01
17	Tubulin beta-2S chain	1.44	0.009	Q13885	11.46	5	49.9	4.89	118.50
18	Clathrin light chain B	1.68	0.005	P09497	8.30	3	25.2	4.64	95.06
19	ATP synthase subunit beta	1.46	0.013	P06576	16.64	16	56.5	5.40	283.06
20	Dynamin-1	1.40	0.006	Q05193	9.61	13	97.3	7.17	242.16

Mass spectrometry protein identification of 2D-DIGE spots of interest and statistical analysis using *t*-test between wild type mice and $APP_{E693\Delta}$-transgenic mice gels ($P < 0.05$). The proteins of mouse hippocampus were separated by 2DE and identified by LC MS/MS, following in-gel digestion with trypsin. The spots representing identified proteins are indicated in Fig. 1D and are designated with their ID accession numbers of Swiss Prot database. Score relates to the probability assignment. Score and sequence coverage were calculated by MASCOT search engine (http://www.matrixscience.com).

Table 2
Functions regulated by proteins that showed an altered expression in $APPE_{693\Delta}$-transgenic mouse hippocampus.

Function	Identified protein	Up/down
Cytoskeletal and their interacting proteins	Neurofilament light polypeptide	Down
	Alpha-internexin	Down
	Actin, cytoplasmic 1	Up/down
	Tubulin β-2A Chain	Up
Chaperone and their interacting proteins	Stress-induced-phosphoprotein 1	Down
	60 kDa heat shock protein	Down
	Heat shock cognate 71 kDa protein	Down
Energy metabolic proteins	Alpha-enolase	Down
	Gamma-enolase	Up
	ATP synthase subunit beta	Up
Vesicle transport and recycling	Dynamin-1	Up
	Clathrin light chain B	Up
Signaling proteins	Protein kinase C and casein kinase substrate in neurons protein 1	Down

The analysis of proteins function was done by using MOTIF (http://www.genome.jp/tools/motif/).

[12]. The amount of dynamin was significantly increased. Our findings in $APP_{E693\Delta}$-transgenic mice without plaque deposition are consistent with previous findings that protein levels of dynamin were increased in Tg2576 mice with plaque deposition [21], suggesting that the release of neurotransmitter is affected by dynamin increased irrespective of AD stage. Also, spot no. 6 was identified as Pacsin 1. The Pacsin 1 is colocalized, oligomerized and bound with dynamin, and both proteins participate in synaptic vesicle endocytosis [17]. The amount of Pacsin 1 was significantly increased. Taken together, Pacsin 1 and dynamin enhanced by Aβ oligomers

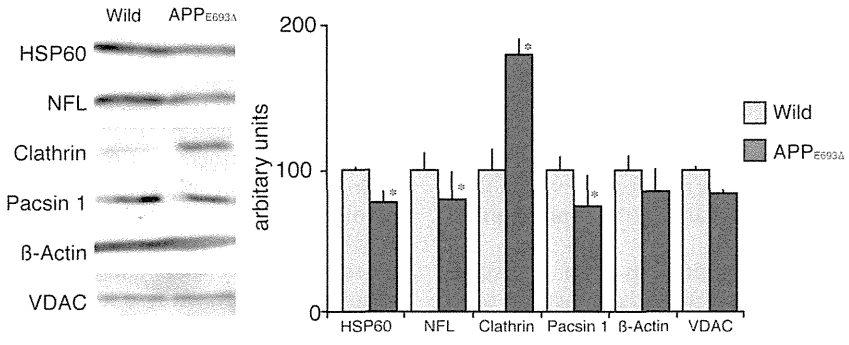

Fig. 2. Differentially expressed proteins validated by Western blotting for the hippocampus of non-transgenic and $APP_{E693\Delta}$-transgenic mice. (A) The levels of HSP60, NFL, clathrin, Pacsin 1, β-actin and VDAC in individual samples of each group were detected. (B) Graphical representation of the semi quantitative analysis (mean ± SEM of O.D. of bands). Data are presented as mean ± SEM ($n = 4$) *t*-test; $^*P < 0.05$ vs. $APP_{E693\Delta}$-transgenic mice.

might change the function of synaptic vesicle in the hippocampus of AD.

Spot no. 18 was identified as clathrin, which is known as the major protein of the polyhedral coat of coated pits and vesicles [7]. The amount of spot no. 18 was significantly decreased. APP was associated clusters of clathrin-coated vesicles and endosomes [3]. Thus, Aβ oligomers might inhibit the vesicle formation by clathrin.

In addition, we performed a validation experiment for HSP60, NFL, clathrin, Pacsin 1 and β-actin as the altered proteins, and VDAC as the unchanged protein (as control) [23]. The increased levels of clathrin, the decreased levels of HSP60, NFL, and Pacsin 1 and the unchanged level of β-actin and VDAC in $APP_{E693\Delta}$-transgenic mice hippocampus were validated by Western blotting (Fig. 2).

In summary, we identified the altered levels of 14 proteins in $APP_{E693\Delta}$-transgenic mice hippocampus using 2D-DIGE and LC–MS/MS approach. This approach elucidated the pathological effects of Aβ oligomers on hippocampus. Our findings might provide a clue for investigation of the hippocampus of AD early stage.

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Annexin A4 is a possible biomarker for cisplatin susceptibility of malignant mesothelioma cells

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ABSTRACT

Mesothelioma is a highly malignant tumor with a poor prognosis and limited treatment options. Although cisplatin (CDDP) is an effective anticancer drug, its response rate is only 20%. Therefore, discovery of biomarkers is desirable to distinguish the CDDP-susceptible versus resistant cases. To this end, differential proteome analysis was performed to distinguish between mesothelioma cells of different CDDP susceptibilities, and this revealed that expression of annexin A4 (ANXA4) protein was higher in CDDP-resistant cells than in CDDP-susceptible cells. Furthermore, ANXA4 expression levels were higher in human clinical malignant mesothelioma tissues than in benign mesothelioma and normal mesothelial tissues. Finally, increased susceptibility was observed following gene knockdown of ANXA4 in mesothelioma cells, whereas the opposite effect was observed following transfection of an ANXA4 plasmid. These results suggest that ANXA4 has a regulatory function related to the cisplatin susceptibility of mesothelioma cells and that it could be a biomarker for CDDP susceptibility in pathological diagnoses.

1. Introduction

Malignant mesothelioma is an aggressive neoplasm located on serosal membrane surfaces such as the pleura, and less frequently the peritoneum, and it has a poor outcome. The five-year survival rate is only about 5%. On the other hand, it is well known that asbestos is the major causative agent in the development of this disease [1–3]. Moreover, malignant mesothelioma takes 40–50 years to develop following exposure to asbestos. Because of its adiabatic potential, asbestos was commonly used as a building material in the 1960–1970s. Thus, an increase in mesothelioma patients is expected in the future. Patients with pleural malignant mesothelioma commonly present with an effusion associated with breathlessness that is often accompanied by chest-wall pain and a cough. After confirming the diagnosis, many patients are treated by intensive multidirectional approaches that combine cytoreductive surgery with intrapleural or intraperitoneal chemotherapy [4–8]. However, cytoreductive surgery is not always possible for patients with extensive intraperitoneal disease. Thus, the role of chemotherapy in malignant mesothelioma is critically important.

CDDP is an extensively used anticancer drug for the treatment of malignant mesothelioma, although the response rate is only about 20% [9–12]. A major problem with CDDP treatment of malignant mesothelioma patients is the development of CDDP insusceptibility. Thus, there is an urgent need to further our understanding of the pathogenesis of malignant mesothelioma, particularly with respect to the expression of proteins that confer drug susceptibility, in order to develop novel therapeutic strategies. In this study, a proteomic analysis was performed using high- and low-CDDP-susceptible malignant mesothelioma cells to identify candidate proteins associated with CDDP susceptibility.

2. Materials and methods

2.1. Cells

H28, H2052, H2452, H226 and MSTO-221H were purchased from American Type Culture Collection and maintained in RPMI1640 medium (Wako) containing 10% fetal calf serum (Biowest). Human mesothelial cells (HMC) were purchased from

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Sciencell and cultured in Mesothelial Cell Growth Medium (ZenBio) under a 5% CO_2 atmosphere at 37 °C.

2.2. Measurement of cisplatin susceptibility in malignant mesothelioma cells

Malignant mesothelioma cells were seeded into 96-well microplates and cultured overnight. Various concentrations of CDDP were added to each well, the plates were incubated for 24 h, and cell viability was measured using Cell count reagent SF (Nacalai tesque). Absorbance was measured using a microplate reader (Bio-Rad) at test and reference wavelengths of 450 and 650 nm, respectively.

2.3. Proteomic analysis using two dimensional differential in-gel electrophoresis

For proteomic analysis, quantitative analysis was performed using two dimensional differential in-gel electrophoresis (2D-DIGE). Cell lysates were prepared from H28 and H2052 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 of 50 μg proteins: 400 pmol Cy3 or Cy5 protein-labeling dye (GE Healthcare Biosciences) in dimethylformamide according to the manufacturer's protocol. The labelled samples were 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) for separation in the first dimension. For the second dimension separation, the IPG-strips were treated with iodoacetamide and applied to SDS–PAGE gels (10% polyacrylamide and 2.7% *N,N′*-diallyltartardiamide gels). After electrophoresis, the gels were scanned with a laser fluoroimager (Typhoon Trio, GE Healthcare Biosciences). The spot-picking gel was scanned after staining with Deep purple total protein stain (GE Healthcare Biosciences). Quantitative analysis of protein spots was carried out with Decyder-DIA software (GE Healthcare Biosciences). For the antigen spots of interest, spots of 1 mm × 1 mm in size were picked using Ettan Spot Picker (GE Healthcare Biosciences).

2.4. In-gel tryptic digestion

Picked gel pieces were destained with 50% acetonitrile/50 mM NH_4HCO_3 for 20 min twice, dehydrated with 75% acetonitrile for 20 min, and then dried using a centrifugal concentrator. Five microliter of 20 μg/ml trypsin (Promega) solution was added to each gel piece and the pieces were incubated for 16 h at 37 °C. The digested peptides were extracted sequentially using 50%, 80%, and 100% acetonitrile and then dried before being suspended in 10 μl of 0.1% formic acid.

2.5. Mass spectrometry and database search

Extracted peptides were analyzed by liquid chromatography ultra high resolution time-of-flight mass spectrometry (LC-UHR TOF-MS/MS; maXis, Bruker Daltonics). The Mascot search engine (http://www.matrixscience.com) was initially used to query the entire theoretical tryptic peptide database as well as SwissProt (http://www.expasy.org/, a public domain database provided by the Swiss Institute of Bioinformatics). The search query assumed the following: (i) the peptides were mono-, di- or tri-isotopic, (ii) methionine residues may be oxidized, (iii) all cysteines were modified with carbamidomethyl.

2.6. Western blot

The cell lysates were separated in 10% SDS–polyacrylamide gels and transferred to Immobilon membranes (Millipore). After blocking by 4% block ace (DS Pharma Biomedical) for 1 h at room temperature, the blots were reacted with primary antibodies in a buffer containing 0.4% block ace, and then with the appropriate peroxidase-conjugated secondary antibodies in the same buffer. Expression of ANXA4 in malignant mesothelioma cells was detected by mouse anti-human ANXA4 (Abnova: 1D3) followed by an HRP-conjugated anti-mouse IgG antibody (Sigma–Aldrich) using the ECL-plus system (GE Healthcare Biosciences). Equal amounts of protein loading were confirmed by parallel β-actin immunoblotting, and signal quantification was performed by densitometric scanning.

2.7. Immunohistochemistry staining

Human mesothelioma and normal tissue sections were deparaffinated in xylene and rehydrated in a graded series of ethanol dilutions. Heat-induced epitope retrieval was performed by incubating at different temperatures following the manufacturer's instructions using Target Retrieval Solution pH 9 (Dako). After heat-induced epitope retrieval treatment, endogenous peroxidase was blocked with a peroxidase blocking reagent (Dako). Following peroxidase blocking, the slides were incubated with 10% bovine serum albumin (BSA) solution for 30 min at room temperature. The slides were then incubated for 60 min with anti-human ANXA4 monoclonal antibody (9 μg/ml) in 3% BSA at room temperature. After washing 3 times with wash buffer (Dako), the slides were incubated for 30 min with ENVISION + Dual Link (Dako) at room temperature. They were then washed final 3 times and stained with 3,3′-diaminobenzidine. After development, the slides were lightly counterstained with Mayer's hematoxylin and mounted with resinous mounting medium.

2.8. Cisplatin susceptibility in cells transfected with ANXA4-siRNA and ANXA4-plasmid

H28 was transfected with ANXA4-siRNA (target sequence: AAGGATATCACAGAAGGATAT, Qiagen) using Hyperfect reagent (Qiagen) according to the manufacturer's instructions. In contrast, H2052 was transfected with ANXA4-pcDNA 3.1 (a gift from Naka T: Laboratory for Immune Signal, National Institute of Biomedical Innovation) using FuGENE HD transfection reagent (Roche). After transfection, the cells were treated with various concentrations of CDDP for 36 h (ANXA4-siRNA) or 24 h (ANXA4-pcDNA 3.1). Cell viability was measured as described above.

2.9. Statistical analysis

Differences in tumor volumes between the control and target groups were compared using the unpaired Student's *t*-test.

3. Results

3.1. CDDP susceptibility in malignant mesothelioma cells

Cell viability following CDDP treatment was examined to determine which cell lines had higher or lower susceptibility to CDDP. Among five tested mesothelioma cell lines, H2052 was the most and H28 the least susceptible cell line (Fig. 1). The IC_{50} values of H28, H2052, H2452, H226 and MSTO-221H were 154.5, 27.8, 66.0, 87.5 and 49.5 μM, respectively.

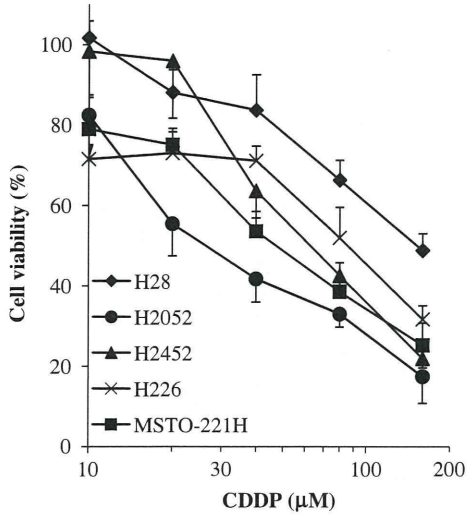

Fig. 1. Susceptibility of malignant mesothelioma cells to CDDP. Mesothelioma cells, H28, H2052, H2452, H226 and MSTO-221H were cultured with various concentrations of CDDP for 24 h 37 °C under 5% CO_2. Cell viability was assayed using the WST-8 assay. Maximal cell viability (100%) was obtained by incubating cells without CDDP. Data are shown as means and standard deviations ($n = 4$).

3.2. Identification of differentially expressed proteins by 2D-DIGE and MS

In order to search for CDDP susceptibility-related proteins, differential proteome analysis between H2052 and H28 cell lines was performed to search for CDDP susceptibility-related proteins (Fig. 2). Quantitative image analysis indicated that a total of eight protein spots representing > 2.0-fold alteration in expression were found and then identified by MS analysis (Table 1). Among those eight proteins, we focused on ANXA4 because this protein plays an important role in membrane stability. Previous reports have indicated that ANXA4 is associated with chemoresistance against platinum-based anticancer drugs in human lung, colon [13] and ovarian cancer [14].

3.3. ANXA4 expression analysis in human malignant mesothelioma cells and mesothelial tissues

Correlations between the expression levels in five malignant mesothelioma cell lines with CDDP-susceptibility were examined using western blot analysis to validate the identified proteins as CDDP susceptibility-related proteins. ANXA4 was expressed at a higher level in H28 cells relative to the other four CDDP-susceptible malignant mesothelioma cell lines (Fig. 3A and B). Expression of ANXA4 in human mesothelial tissue was analyzed by immunohistochemistry staining with an anti-human ANXA4 monoclonal antibody. Fig. 3C indicates that ANXA4 was expressed at higher levels in human malignant mesothelioma tissues than in benign mesothelioma tissues and normal mesothelial tissues.

3.4. Gene regulation of ANXA4 in malignant mesothelioma cells by knockdown and overexpression

ANXA4-siRNA and ANXA4-pcDNA 3.1 were next transfected to H28 and H2052 before CDDP treatment to evaluate correlations between ANXA4 expression levels and CDDP susceptibility. The IC_{50} values of [H28/non treat: H28/control-siRNA: H28/ANXA4-siRNA] were [80.0 μM: 71.8 μM: 15.5 μM] and [H2052/control-pcDNA 3.1: [H2052/ANXA4-pcDNA 3.1] were [55.2 μM: 89.7 μM], respectively (Fig. 4A–D). These results suggested that the CDDP susceptibility of H28 cells was increased by ANXA4-siRNA transfection and that of H2052 cells was decreased by ANXA4-pcDNA 3.1 transfection.

4. Discussion

In this study, a proteomic analysis was performed based on 2D-DIGE using malignant mesothelioma cell lines to identify candidate proteins associated with CDDP susceptibility (Figs. 1 and 2). Eight proteins that were differentially expressed in H28 cells compared with H2052 cells were identified (Table 1). ANXA4 was found to be expressed at a higher level in H28 cells relative to levels in CDDP-susceptible malignant mesothelioma cells by western blot

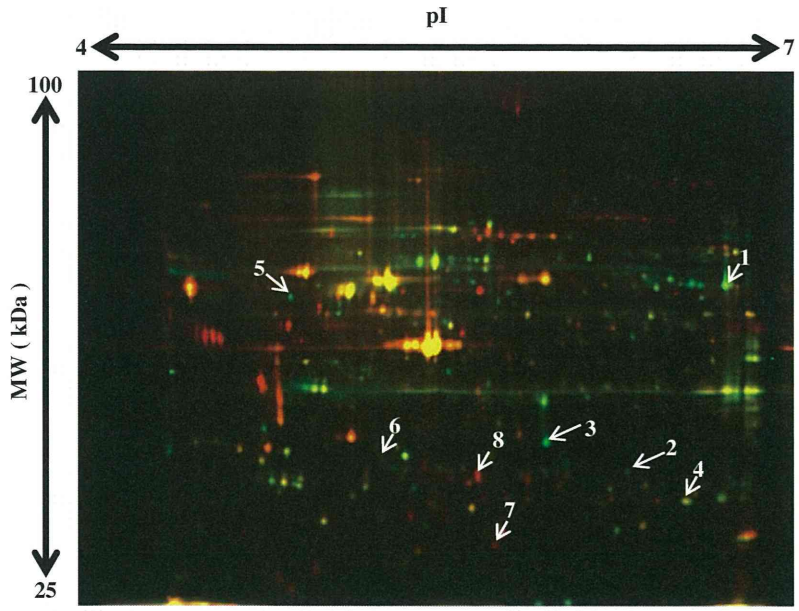

Fig. 2. 2D-DIGE image of fluorescently labeled proteins from human mesothelioma cell lines H28 and H2052. Proteins from high- and low-susceptible mesothelioma cells (H2052, H28) were labeled with cy3 and cy5, respectively, and 2D electrophoresis was performed. The differentially expressed spots in H28 indicated by white arrows were then identified by LC-TOF-MS/MS. Table 1 contains additional information about the identified proteins.

Table 1
Proteins expressed at higher or lower levels in H28 compared to H2052.

No.	Accession number	Protein name	pI	MW (kDa)	Expression ratio (H28/H2052)
1	P11413	Glucose-6-phosphate 1-dehydrogenase	6.4	59.3	21.0
2	P78417	Glutathione *S*-transferase omega-1	6.2	27.6	7.4
3	P09525	Annexin A4	5.6	35.9	3.6
4	P30041	Peroxiredoxin-6	6.0	25.0	3.5
5	Q09028	Histone-binding protein RBBP4	4.7	47.7	3.0
6	P07195	L-lactate dehydrogenase B chain	5.7	36.6	2.9
7	P32119	Peroxiredoxin-2	5.7	21.9	0.03
8	Q9Y696	Chloride intracellular channel protein 4	5.5	28.8	0.13

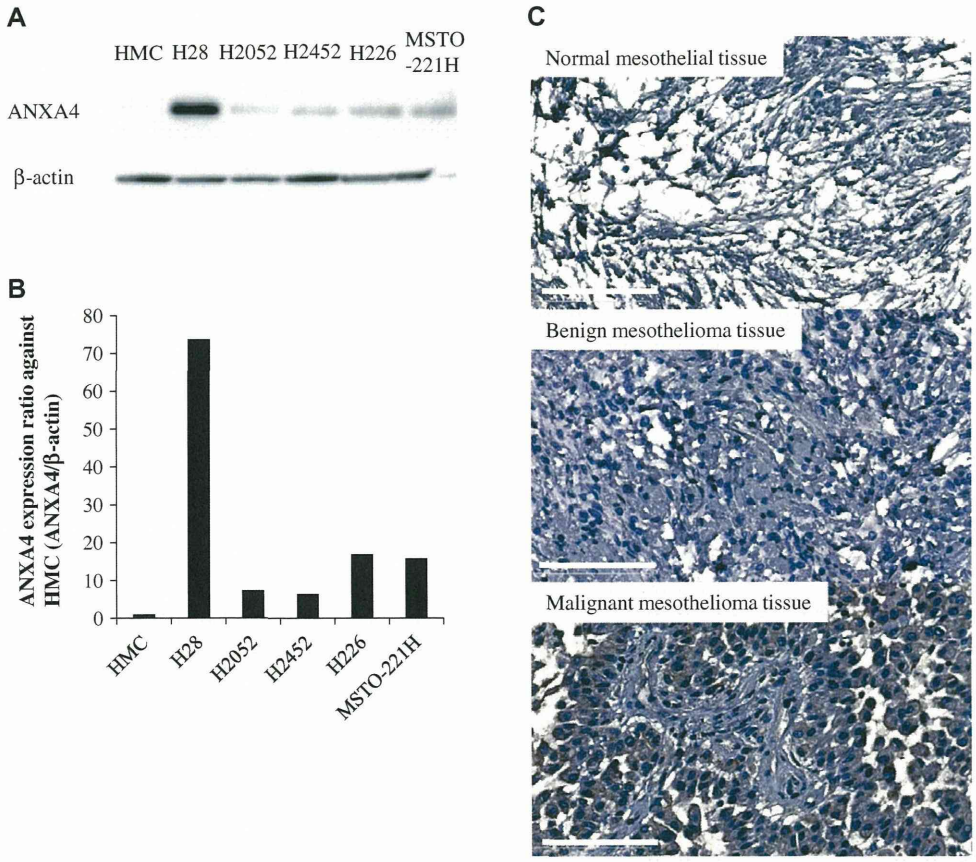

Fig. 3. ANXA4 expression analysis in human malignant mesothelioma cells and mesothelial tissues. ANXA4 expression levels in human primary mesothelial cells, HMC, and mesothelioma cell lines (H28, H2052, H2452, H226, MSTO-221H) were analyzed by western blotting (A). Intensity of the western blotting images was quantified by densitometry (B). Expression of ANXA4 in human mesothelial tissues was analyzed by immunostaining using an anti-human ANXA4 antibody (C). Top, middle and bottom panels are normal mesothelial, benign and malignant mesothelioma tissues, respectively. The tissue sections were counterstained using hematoxylin. Representative 400 × photomicrographs presented (bar: 100 μm).

analysis (Fig. 3A and B). Furthermore, ANXA4 was expressed in malignant mesothelioma tissue but not in benign mesothelial tumor and normal mesothelial tissues (Fig. 3C). Thus, ANXA4 was expressed in CDDP-susceptible malignant mesothelioma cells and specifically in malignant mesothelioma tissues. These results indicate that ANXA4 expression in malignant mesothelioma cells may be correlated with CDDP susceptibility, although this relationship must be validated in future studies of human clinical malignant mesothelial cases. The CDDP susceptibility of H28 cells was actually increased by ANXA4 knockdown, and that of H2052 cells was decreased by ANXA4 overexpression (Fig. 4). Thus, these results suggest that ANXA4 plays an important role in chemoresistance against CDDP.

ANXA4 has already been characterized as a regulator of cell membranes with calcium dependency [15–17]. Recently, some studies have reported the protein is associated with membrane permeability [18], ion channels [19] and exocytosis [20,21]. These observations may explain in part the correlation of ANXA4 with modulation of drug susceptibility in cancer cells.

This study demonstrates for the first time elevated ANXA4 protein expression in malignant mesothelioma cells that have less susceptibility to CDDP. *In vitro* evaluation of drug susceptibility against CDDP in malignant mesothelioma cells derived from cancer patients would be important in clinical conditions because doctors as well as patients wish to avoid treatment with inefficacious drugs. Consequently, the susceptibility of a given patient against CDDP could be confirmed by analyzing the expression level of ANXA4 in malignant mesothelioma patients at the time of diagnosis. Furthermore, if ANXA4 expression could be blocked specifically in malignant mesothelioma cells by nucleic acid drugs such as siRNA, this procedure would prove useful in clinical situations involving CDDP treatment. The present study may contribute to