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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.

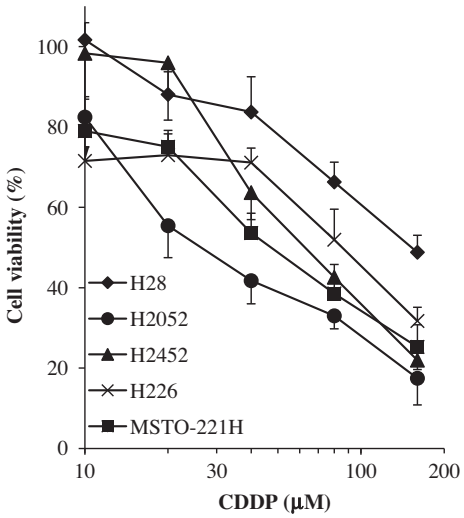

Fig. 1. Susceptibility of malignant mesothelioma cells to CDDP. Mesothelioma cells, H28, H2052, H2452, H226 and MSTO-221H were cultured with various concentrations of CDDP for 24 h 37 °C under 5% CO_2. Cell viability was assayed using the WST-8 assay. Maximal cell viability (100%) was obtained by incubating 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

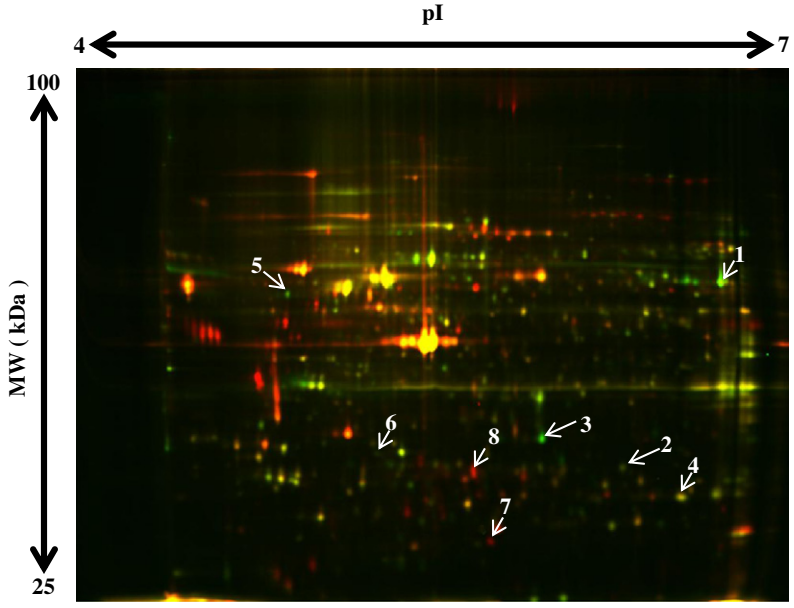

Fig. 2. 2D-DIGE image of fluorescently labeled proteins from human mesothelioma cell lines H28 and H2052. Proteins from high- and low-susceptible mesothelioma cells (H2052, H28) were labeled with cy3 and cy5, respectively, and 2D electrophoresis was performed. The differentially expressed spots in H28 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	p*I*	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

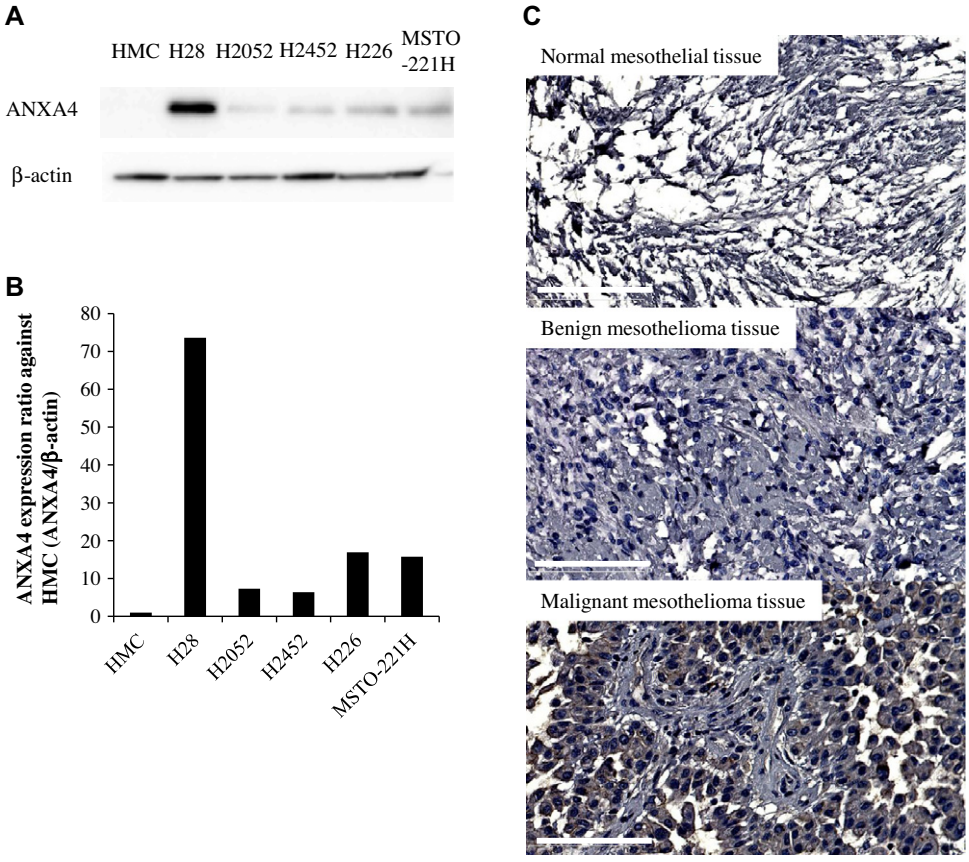

Fig. 3. ANXA4 expression analysis in human malignant mesothelioma cells and mesothelial tissues. ANXA4 expression levels in human primary mesothelial cells, HMC, and mesothelioma cell lines (H28, H2052, H2452, H226, MSTO-221H) were analyzed by western blotting (A). Intensity of the western blotting images 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

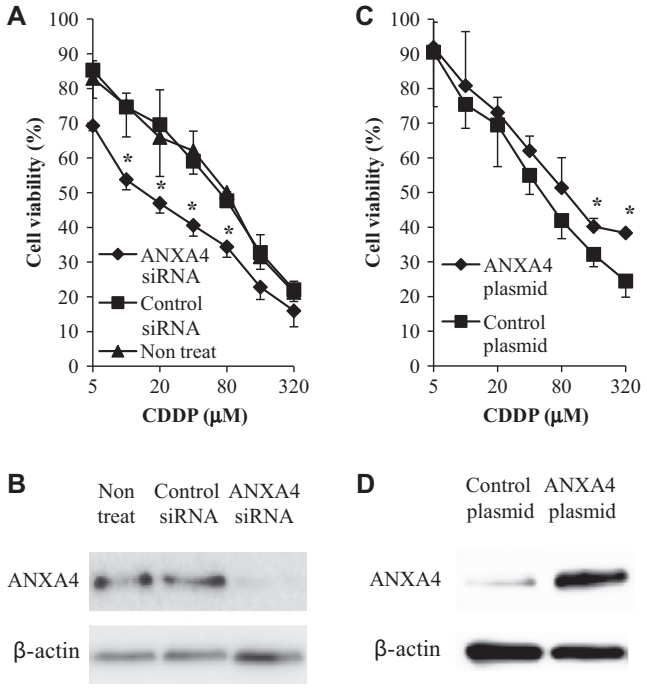

Fig. 4. The effect of ANXA4 gene knockdown and overexpression on CDDP susceptibility in malignant mesothelioma cells. Transfection of ANXA4 siRNA or plasmid into malignant mesothelioma cells confers resistance to CDDP. Cell survival after 24 h treatment of H28/ANXA4 siRNA or H2052/ANXA4 plasmid with different concentrations of CDDP (A and C). Expression of ANXA4 was analyzed by western blot analysis (B and D). Data are shown as means and standard deviations ($n = 4$). $^{*}P < 0.05$ (Control siRNA or plasmid vs. ANXA4 siRNA or plasmid).

establishment of a new therapeutic strategy for malignant mesothelioma patients by suggesting a novel diagnostic and therapeutic target.

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