

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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Rho GDP-dissociation inhibitor alpha is associated with cancer metastasis in colon and prostate cancer

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Since metastasis is one of the most important prognostic factors in colorectal cancer, development of new methods to diagnose and prevent metastasis is highly desirable. However, the molecular mechanisms leading to the metastatic phenotype have not been well elucidated. In this study, a proteomics-based search was carried out for metastasis-related proteins in colorectal cancer by analyzing the differential expression of proteins in primary versus metastasis focus-derived colorectal tumor cells. Protein expression profiles were determined using a tissue microarray (TMA), and the results identified Rho GDP-dissociation inhibitor alpha (Rho GDI) as a metastasis-related protein in colon and prostate cancer patients. Consequently, Rho GDI may be useful as a diagnostic biomarker and/or a therapeutic to prevent colon and prostate cancer metastasis.

1. Introduction

Colorectal cancer is known as a major metastatic cancer, and 40–50% of patients already have a metastatic focus at presentation. Moreover, the 5-year survival of these patients is under 10% (Davies et al. 2005). Thus, metastasis is one of the most important prognostic factors in colorectal cancer. In order to improve rates of cancer remission, it will be necessary to clarify the detailed molecular mechanisms of cancer metastasis and to utilize this information to establish new diagnostic and therapeutic techniques. Many researchers have searched for metastasis-related molecules (Liu et al. 2010; Shuehara et al. 2011) using proteomics techniques (Hanash 2003). Comprehensive mapping of the molecular changes during metastasis would greatly improve our understanding of the recurrence and management of cancer. However, the knowledge gained so far in these studies has not been sufficient to improve cancer remission rates.

Here we show the potential of Rho GDI as a metastasis-related protein in colon and prostate cancer patients. In order to identify metastasis-related proteins, the protein expression patterns of human colorectal cancer cells with different metastatic characters were compared. Because these cells were derived from the same patient (SW480: a surgical specimen of a primary colon adenocarcinoma, SW620: a lymph node metastatic focus), cancer metastasis-related protein candidates could be effectively sought without background variations due to differences between individuals. Furthermore, by analyzing the expression of candidate proteins in many clinical samples using a TMA, we attempted to validate the association of these candidates

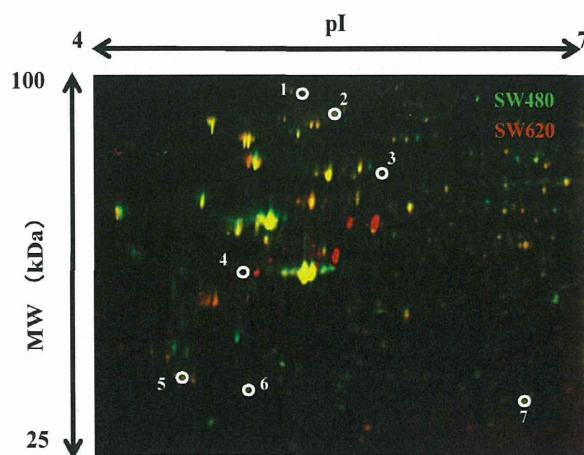


Fig. 1: 2D-DIGE image of fluorescently-labeled proteins from different metastatic human colorectal cancer cells. SW480 is human colorectal cancer cell line derived from a primary tumor and SW620 is derived from a metastatic focus from the same patient. Proteins from the colon cancer cells (SW480, SW620) were labeled with Cy3 and Cy5 respectively, and analyzed by 2D electrophoresis. The differentially-expressed spots (white circles) were then identified by LC-UHR TOF/MS

with metastasis. TMA is a slide glass containing many clinical tissues, and it enables one to carry out a high-throughput analysis by evaluating the relationship between expression profiles of each candidate molecule and clinical information such as metastasis. (Imai et al. 2011; Yoshida et al. 2011).

Table 1: High expression proteins in SW620 compared to SW480

	Accession	Protein name	MW (kDa)	pI	Ratio (SW620 / SW480)
1	P12109	collagen alpha-1(VI) chain	108.6	5.3	1.53
2	Q15459	splicing factor 3A subunit 1	88.9	5.2	1.61
3	P13797	T-plastin	70.9	5.5	1.59
4	P60709	actin cytoplasmic 1	42.1	5.3	1.50
5	P63104	14-3-3 zeta/delta	27.9	4.7	1.63
6	P52565	Rho GDP-dissociation inhibitor 1 (Rho GDI)	23.3	5.0	1.90
7	P30041	Peroxiredoxin-6 (PRDX6)	25.1	6.0	1.86

2. Investigations, results and discussion

In order to search for metastasis-related proteins, we analyzed differentially-expressed proteins between SW480 and SW620 by two-dimensional differential in-gel electrophoresis (2D-DIGE) (Fig. 1). As a result, 7 spots with at least a 1.5-fold-altered expression level were found by quantitative analysis, and these spots were identified by mass spectrometry (Table 1). Three molecules having a high SW620/SW480 expression ratio indicating a strong association with cancer metastasis were identified: Rho GDP-dissociation inhibitor alpha (Rho GDI), peroxiredoxin-6 (PRDX6) and 14-3-3 zeta/delta.

The expression profiles of these proteins were analyzed by immunohistochemistry using the TMA with colon cancer and multiple cancer tissues. Results of this analysis indicated that expression of PRDX6 and 14-3-3 zeta/delta had no relationship to the clinical status of cancer metastasis (data not shown). On the other hand, in positive cases of lymph node metastasis, the expression ratio of Rho GDI was significantly higher than in the negative cases. Furthermore, the same trend was seen when tissues from prostate cancer patients were analyzed (Table 2). To confirm these results, the expression levels of Rho GDI protein in colon cancer cell lines with different metastatic potential (SW480 < SW620 < SW620-OK1 < SW620-OK2: Characteristics of SW620-OK1 and SW620-OK2 are described in *Experimental*) were investigated by western blot analysis (Fig. 2). The expression of Rho GDI was found to be up-regulated with the development of metastatic characteristics. These results suggested that Rho GDI is correlated with cancer metastasis.

Rho GDI has been identified as key regulator of Rho family GTPases. Activation of growth factor receptors and integrins can promote the exchange of GDP for GTP on Rho proteins (Bishop et al. 2000). Furthermore, GTP-bound Rho proteins interact with a range of effector molecules to modulate their activity or localization, and this leads to changes in cell behavior. It is clear that Rho family GTPases are involved in the control of cell morphology and motility (Etienne-Manneville et al. 2002; Hall et al. 1997; Van Aelst et al. 1997). The importance of Rho protein and Rho GDI in cancer progression, particularly in the area of metastasis, is becoming increasingly evident. Recently, some reports have indicated that the expression of Rho GDI was correlated with colorectal and breast cancer metastasis (Zhao et al. 2008; Kang et al. 2010). Thus, our findings are consistent with these reports and further suggest that the expression of Rho GDI is also correlated with prostate cancer metastasis. Consequently, Rho GDI should be considered as a diagnostic marker or new therapeutic target for cancer metastasis.

3. Experimental

3.1. Cell lines

SW480 is a human colorectal cancer cell line derived from a primary focus and SW620 is derived from a metastatic focus of the same patient. These

cells were purchased from American Type Culture Collection and maintained at 37 °C using Leibovitz's L-15 medium (Wako) supplemented with 10% FCS. SW620-OK1 and -OK2 were established by the following procedure: 1×10^6 SW620 cells were injected into the spleens of nu/nu mice. After 8 weeks, SW620-OK1 was established from a liver metastatic focus. Furthermore, SW620-OK2 was established from SW620-OK1 using the same procedures.

3.2. 2D-DIGE analysis

Cell lysates were prepared from SW480 and SW620 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. Briefly, the labelled samples were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% Pharylyte (GE Healthcare Biosciences)) and applied to a 24-cm immobilized pH gradient gel strip (IPG-strip pH 4–7 NL) for separation in the first dimension. Samples for the spot-picking gel were prepared without labelling by Cy-dyes. For the second dimension separation, the IPG-strips were applied to SDS-PAGE gels (10% polyacrylamide and 2.7% N,N'-diallyltartardiamide gels). After electrophoresis, the gels were scanned with a laser fluorimager (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).

3.3. In-gel tryptic digestion

Picked gel pieces were digested with trypsin as described below. The 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. Next, 5 µl of 20 µl/ml trypsin (Promega) solution was added to each gel piece and incubated for 16 h at 37 °C. Three solutions were used to extract the resulting peptide mixtures from the gel pieces. First, 50 µl of 50% (v/v) acetonitrile in 0.1% (v/v) formic acid (FA) was added to the gel pieces, which were then sonicated for 5 min. Next, we collected the solution and added 80% (v/v) acetonitrile in 0.1% FA. Finally, 100% acetonitrile was added for the last extraction. The peptides were dried and then re-suspended in 10 µl of 0.1% FA.

3.4. Mass spectrometry and database search

Extracted peptides were analyzed by liquid chromatography Ultra High Resolution time-of-flight mass spectrometry (LC-UHR TOF/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 pro-

Table 2: Expression profile of Rho GDI in primary cancers with or without lymph node metastasis

	Number of Rho GDI positive cases (positive ratio)	
	in metastasis negative cases	in metastasis positive cases
Colon cancer*	11/14 (79%)	19/19 (100%)
Prostate cancer*	18/23 (78%)	11/11 (100%)

* $p < 0.05$: Mann Whitney U test

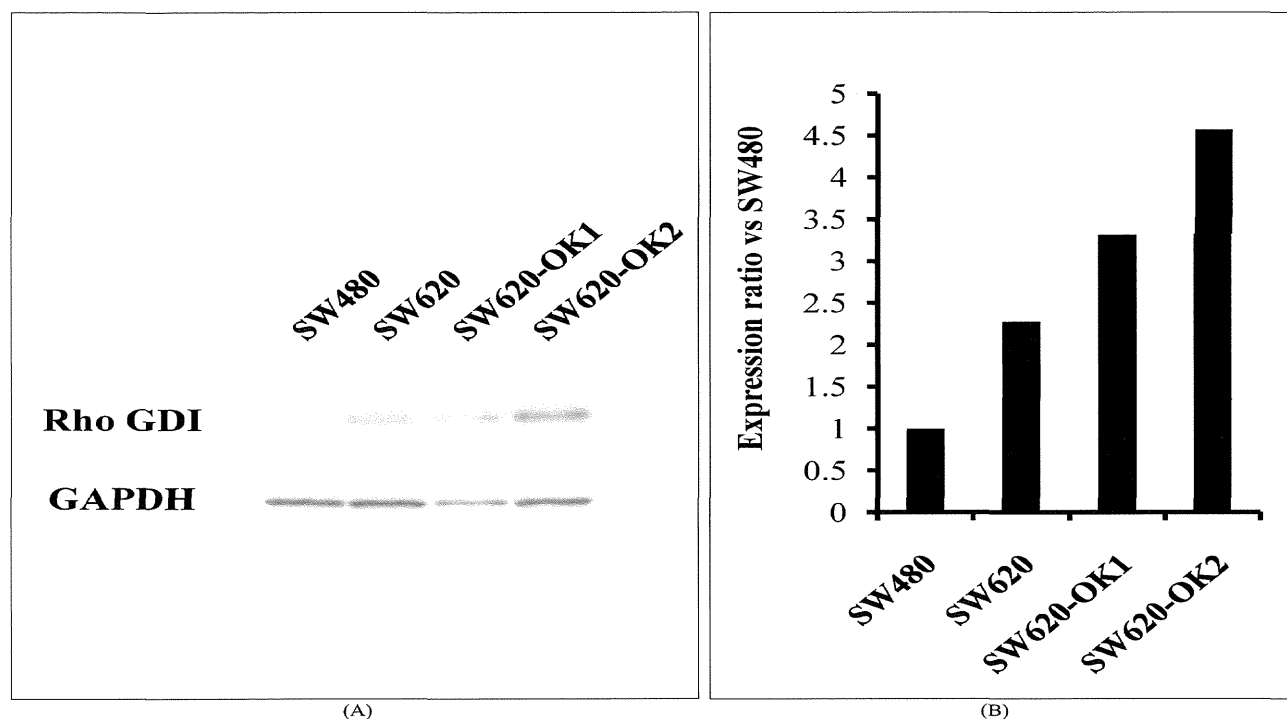


Fig. 2: Rho GDI expression levels in colon cancer cell lines with different metastatic abilities. Rho GDI expression levels in colon cancer cell lines (SW480, SW620, SW620-OK1, SW620-OK2) analyzed by western blotting (A). SW620-OK1, SW620-OK2 have been established as high metastatic sub-lines of SW620 using a mouse metastasis model. Intensity of the western blotting images was quantified by densitometry (B)

vided by the Swiss Institute of Bioinformatics). The search query assumed the following: (i) the peptides were monoisotopic (ii) methionine residues may be oxidized (iii) all cysteines are modified with iodoacetamide.

3.5. TMA Immunochemical staining

TMA slides with human colon cancer samples or multiple cancer samples (Biomax) were de-paraffinated in xylene and rehydrated in a graded series of ethanol washes. Heat-induced epitope retrieval was performed while maintaining the Target Retrieval Solution pH 9 (Dako) at the desired temperature according to manufacturer's instructions. After the treatment, endogenous peroxidase was blocked with 0.3% H₂O₂ in Tris-buffer saline (TBS) for 5 min. After washing twice with TBS, TMA slides were incubated with 10% BSA blocking solution for 30 min. The slides were then incubated with the anti-Rho GDI (Santa Cruz Biotechnology) for 60 min. After washing three times with wash buffer (Dako), each series of sections was incubated for 30 min with Envision + Dual Link (Dako). The reaction products were rinsed twice with wash buffer and then developed in liquid 3, 3'-diaminobenzidine (Dako) for 3 min. After the development, sections were counterstained with Mayer's hematoxylin. All procedures were performed using AutoStainer (Dako).

3.6. TMA Immunohistochemistry scoring

The optimized staining conditions for TMAs corresponding to human colon as well as multiple cancers were determined based on the co-existence of both positive and negative cells in the same tissue sample. Signals were considered positive when reaction products were localized in the expected cellular component. The criteria for scoring of stained tissues were as follows: the distribution score was 0 (0%), 1 (1–50%) or 2 (51–100%), indicating the percentage of positive cells among all tumor cells present in one tissue. The intensity of the signal (intensity score) was scored as 0 (no signal), 1 (weak), 2 (moderate) or 3 (marked). The distribution and intensity scores were then summed into a total score (TS) of TS0 (sum=0), TS1 (sum=2), TS2 (sum=3), and TS3 (sum=4–5). Throughout this study, TS0 or TS1 was regarded as negative, whereas TS2 or TS3 were regarded as positive.

3.7. Western Blot

Expression of Rho GDI in colon cancer cells was detected by anti-Rho GDI (Santa Cruz Biotechnology) and HRP conjugated anti-mouse IgG antibody (Sigma) using the ECL-plus system. Equal amounts of protein loading were confirmed by parallel β -actin immunoblotting, and signal quantification was performed by densitometric scanning.

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