

# Proteomics-based Identification of RS/DJ-1 as a Novel Circulating Tumor Antigen in Breast Cancer<sup>1</sup>

François Le Naour, David E. Misek,  
Melissa C. Krause, L. Deneux,  
Thomas J. Giordano, S. Scholl, and  
Samir M. Hanash<sup>2</sup>

Departments of Pediatrics [F. L. N., D. E. M., M. C. K., S. M. H.] and Pathology [T. J. G.], University of Michigan, Ann Arbor, Michigan 48109, and Curie Institute, Medical Section, Paris, France [L. D., S. S.]

## ABSTRACT

**Purpose:** We used a proteomics-based approach to identify tumor proteins that elicit a humoral response in breast carcinoma and that may occur as circulating antigens.

**Experimental Design:** The breast cell line SUM-44 was used as a source of tumor cell proteins for two-dimensional PAGE (2-D PAGE) and for Western blot analysis in which individual sera were analyzed for primary antibodies.

**Results:** Sera from 30 newly diagnosed patients with breast cancer were screened for IgG antibodies to tumor cell proteins. Sera from 116 patients with other cancers and from 25 healthy subjects served as controls. Restricted reactivity against a set of three proteins, identified by mass spectrometry as isoforms of a novel oncogenic protein that regulates RNA-protein interaction (designated RS/DJ-1), was observed in four patients with breast cancer, but not in healthy subjects. The identity was further confirmed by Western blotting with specific antibodies. RS/DJ-1 was found to be secreted in the breast cell line SUM-44, which led us to determine whether RS/DJ-1 was found in circulation in breast cancer. Interestingly, unlike in controls, RS/DJ-1 was readily detectable in sera from 37% of newly diagnosed patients with breast cancer.

**Conclusion:** The presence of autoantibodies and/or circulating RS/DJ-1 protein in sera from patients with breast cancer may have clinical utility.

Received 2/15/01; revised 7/12/01; accepted 8/9/01.

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<sup>1</sup> F. Le Naour was the recipient of a grant from the "Association pour la Recherche contre le Cancer" (ARC) and from the "Institut National de la Santé et de la Recherche Médicale" (INSERM).

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Pediatrics, University of Michigan Medical School, Box 0656, MSRB I, Room A520C, Ann Arbor, MI 48109-0656. Phone: (734) 763-9311; Fax: (734) 647-8148; E-mail: shanash@umich.edu.

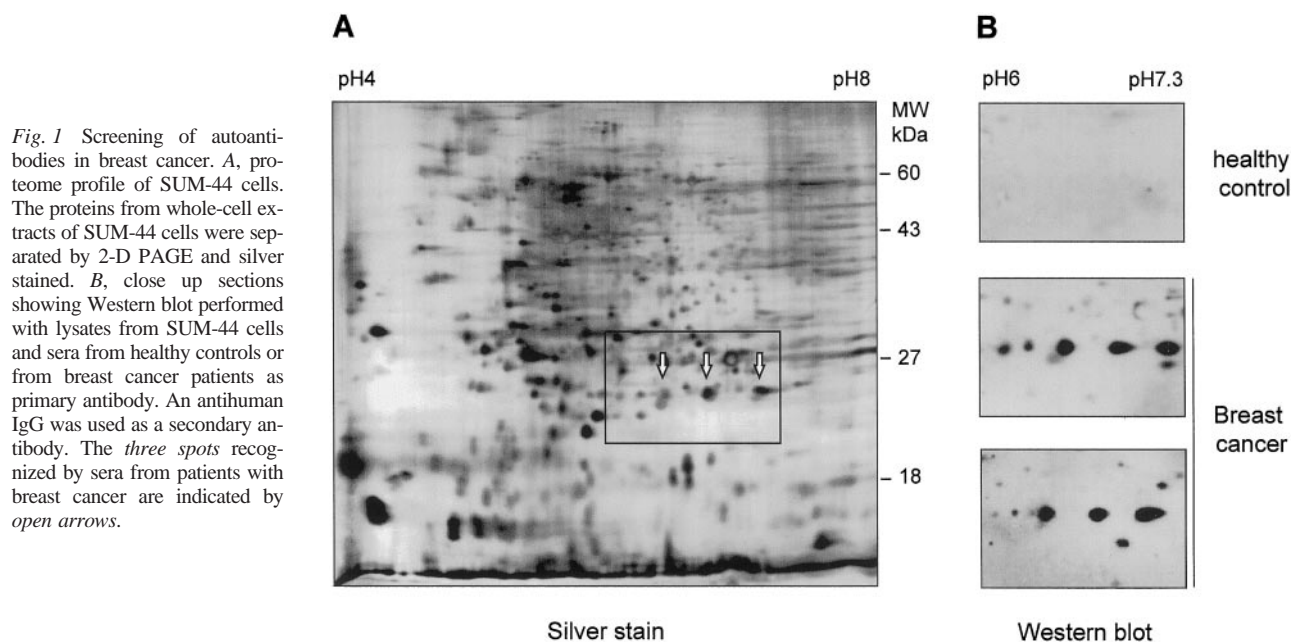
## INTRODUCTION

There is at present much interest in identifying markers for the detection of breast cancer. We have implemented a proteomics-based approach to identify tumor markers based on their occurrence as tumor antigens that elicit a humoral response during tumorigenesis. There is substantial evidence for a humoral immune response to cancer in humans, as demonstrated by the identification of antibodies against a number of intracellular and surface antigens in patients with various tumor types (1–3).

In breast cancer, autoimmunity has been shown against several proteins, such as p53, heat shock protein 90, c-erbB-2/HER2/neu, and mucin-related antigens (4–10). The presence of p53 autoantibodies has been observed in 15% of patients with breast cancer and has been shown to be associated with a bad prognosis (8, 10). Autoantibodies to the 90-kDa heat shock protein have also been associated with poor survival in breast cancer (7, 11). The proto-oncogene c-erbB-2/HER2/neu, which encodes a growth factor receptor, is overexpressed in 20–30% of patients with breast cancer (12). The presence of c-erbB-2/HER2/neu autoantibodies has been observed in 11% of cases and has been found to correlate with overexpression of the protein in tumor tissue (13). Elevated c-erbB-2/HER2/neu protein levels have been found in the serum of 29% of patients with breast carcinoma and have been associated with poor outcome (14, 15). On the other hand, the presence of MUC1 autoantibodies has been associated with a reduced risk for disease progression in patients with breast cancer (5). The mucin protein MUC1, a transmembrane glycoprotein involved in cell-cell and cell-extracellular matrix interactions, has been shown to be frequently overexpressed or/and underglycosylated in malignant breast cancer (16, 17). At present, MUC1/CA 15-3 is used as a circulating marker for breast cancer. The CA 15-3 concentration at initial presentation has prognostic significance. Serial measurements have the potential to both detect recurrences preclinically and monitor the treatment of metastatic breast cancer (17).

We have implemented a proteomics-based approach for the identification of tumor antigens that elicit a humoral response. To this end, we used 2-D<sup>3</sup> PAGE to simultaneously separate several thousand individual cellular proteins from tumor tissue or tumor cell lines. Separated proteins were transferred onto membranes, and sera from cancer patients were screened individually by Western blot analysis for antibodies that react against separated proteins. Proteins that specifically reacted with sera from cancer patients were identified by mass spectrometric

<sup>3</sup> The abbreviations used are: 2-D, two-dimensional; RS, RNA-binding protein regulatory subunit; IHC, immunohistochemistry; PVDF, polyvinylidene difluoride; 1-D, one-dimensional; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NCBI, National Center for Biotechnology Information; RBS, RNA-binding subunit.



**Fig. 1** Screening of autoantibodies in breast cancer. **A**, proteome profile of SUM-44 cells. The proteins from whole-cell extracts of SUM-44 cells were separated by 2-D PAGE and silver stained. **B**, close up sections showing Western blot performed with lysates from SUM-44 cells and sera from healthy controls or from breast cancer patients as primary antibody. An antihuman IgG was used as a secondary antibody. The three spots recognized by sera from patients with breast cancer are indicated by open arrows.

analysis and/or amino acid sequencing. In this study, we report the identification using a proteomics-based approach using autoantibodies of the IgG class directed against the oncogenic protein RS/DJ-1 in sera from 4 of 30 patients with breast cancer. Circulating RS/DJ-1 antigen was readily detectable in sera from 11 of 30 newly diagnosed patients with breast cancer.

## MATERIALS AND METHODS

**Sera and Tumor Tissues.** Sera were obtained at the time of diagnosis from 30 patients with breast cancer, after informed consent was given. Sera from 25 healthy individuals and from 116 patients with other cancers (including 54 with lung cancer, 46 with liver cancer, and 16 with brain tumors) were used as controls. Fresh tumor tissue was obtained at the time of diagnosis (*i.e.*, biopsy tissue) from patients with breast cancer. After excision, the tumor tissue was immediately frozen at  $-80^{\circ}\text{C}$ . Twenty cases of invasive breast carcinoma were randomly selected from the University of Michigan pathology archives for RS IHC.

**Cell Culture, Preparation of Secreted Proteins, and Radioactive Labeling.** The human breast cancer cell line SUM-44 was developed from a pleural effusion from a patient with estrogen receptor-positive breast cancer and was harvested as described previously (18). The cells were cultured at  $37^{\circ}\text{C}$  in a 6%  $\text{CO}_2$ -humidified incubator in a complete serum-free medium. This medium consisted of Ham's F-12 supplemented with bovine serum albumin (1 mg/ml), ethanolamine (5 mM), HEPES (10 mM), transferrin (5  $\mu\text{g}/\text{ml}$ ), triiodothyronine (10  $\mu\text{M}$ ), sodium selenite (50  $\mu\text{M}$ ), insulin (5  $\mu\text{g}/\text{ml}$ ), hydrocortisone (1  $\mu\text{g}/\text{ml}$ ), gentamicin (5  $\mu\text{g}/\text{ml}$ ), and fungizone (500 ng/ml). The medium was changed three times a week, and the cells were reseeded at a split ratio of 1:3 after they had reached 70–80% confluence. The cell lines A549 (lung), U87 (glioma), and HuH7 (liver) were harvested in DMEM supplemented with 10%

FCS. All cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY) or Sigma (St. Louis, MO). For preparation of secreted proteins, cultured cells were harvested in fresh regular medium for 14 h. The supernatant was concentrated in a Centricon YM-3 (Millipore) to a final volume of 100  $\mu\text{l}$  according with the instructions of the manufacturer and stored at  $\sim 80^{\circ}\text{C}$ .  $^{32}\text{P}$   $\text{P}_i$  labeling was performed by preincubating the cells for 2 h with 200  $\mu\text{Ci}/\text{ml}$  of  $[^{32}\text{P}]\text{O}_4^{3-}$  (Amersham, Arlington Heights, IL) in phosphate-free culture medium (Life Technologies).

**2-D PAGE.** The procedure followed was as described previously (19). Cultured cells and tumor and nontumor tissues were solubilized in lysis buffer containing 9.5 M urea (Bio-Rad, Rockville Center, NY), 2% NP40, 2% carrier ampholytes (pH 4–8; Gallard/Schlessinger, Carle Place, NY), 2%  $\beta$ -mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured by the Bradford assay (Bio-Rad, Hercules, CA). Proteins (175  $\mu\text{g}$ ) were applied to isoelectric focusing gels. Isoelectric focusing was conducted with pH 4–8 carrier ampholytes at 700 V for 16 h, followed by 1000 V for an additional 2 h. The first-dimension gel was loaded on the second-dimension gel after equilibration in second-dimension sample buffer [125 mM Tris (pH 6.8) containing 10% glycerol, 2% SDS, 1% DTT, and bromphenol blue]. For the second-dimension separation, a gradient of 11–14% acrylamide (Serva, Crescent Chemical, Hauppauge, NY) was used. Proteins were transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA) or visualized by silver staining of the gels (19, 20). Serum proteins were separated by 1-D PAGE on a 10% acrylamide gel under nonreducing conditions.

**Western Blotting.** After transfer, membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mM Tris-HCl (pH 7.5), 2.5 mM EDTA (pH 8), 50 mM NaCl. The membranes were incubated for 2 h at room temperature with

Table 1 Clinical characteristics of subjects with breast cancer

Patient	Age (yrs)	Stage	Pathology	RS Ab <sup>a</sup>	RS Ag <sup>-</sup>
1	38	T <sub>2</sub> N <sub>1</sub> M <sub>0</sub>	Infiltrat. ductal	Ab+	Ag+
2	50	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
3	54	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
4	57	T <sub>3</sub> N <sub>1b</sub> M <sub>0</sub>	DI	Ab-	Ag-
5	84	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	Mucinous	Ab-	Ag+
6	72	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
7	52	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
8	78	T <sub>4d</sub> N <sub>3</sub> M <sub>0</sub>	DI	Ab-	Ag-
9	86	T <sub>3</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag+
10	37	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	Infiltrat. lobula	Ab-	Ag-
11	72	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
12	47	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab+	Ag+
13	72	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	Infiltrat. lobula	Ab-	Ag-
14	72	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
15	42	T <sub>2</sub> N <sub>1</sub> M <sub>1</sub>	DI	Ab-	Ag-
16	49	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag+
17	62	Recurrence	Infiltrat. lobula	Ab-	Ag-
18	66	T <sub>0</sub> N <sub>0</sub> M <sub>0</sub>	Intraductal	Ab-	Ag+
19	65	Preinvasive	Intraductal	Ab-	Ag+
20	56	T <sub>4</sub> N <sub>1a</sub> M <sub>1</sub>	DI	Ab-	Ag-
21	46	T <sub>1</sub> N <sub>0</sub>	DI	Ab-	Ag+
22	55	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag+
23	45	T <sub>2</sub> N <sub>0</sub>	DI	Ab-	Ag-
24	65	T <sub>3</sub> N <sub>0</sub> M <sub>3</sub>	DI	Ab-	Ag-
25	64	T <sub>3</sub> N <sub>1a</sub> M <sub>0</sub>	DI	Ab+	Ag+
26	63	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-
27	77	T <sub>2</sub> N <sub>0</sub>	DI	Ab-	Ag-
28	87	T <sub>2</sub> N <sub>x</sub> M <sub>0</sub>	DI	Ab-	Ag-
29	75		DI	Ab+	Ag+
30	40	T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	DI	Ab-	Ag-

<sup>a</sup> Ab, antibody; Ag, antigen; DI, Infiltrat. ductal.

serum obtained from either patients or healthy individuals as a source of primary antibody at a 1:300 dilution, with the rabbit polyclonal antibody against RS (kindly provided by Dr. Hod) at a 1:5000 dilution, or with the mouse monoclonal antibody against DJ-1 (StressGen, Victoria, Canada) at 0.5 µg/ml. Mouse monoclonal anti-β-tubulin DM 1A (Sigma) was used at a dilution of 1:1000. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated antihuman, antimouse, or antirabbit (Amersham) IgG antibodies at a dilution of 1:1000. Immunodetection was accomplished by enhanced chemiluminescence (Amersham) followed by autoradiography on hyperfilm MP (Amersham). The horseradish peroxidase-conjugated mouse antihuman IgG1, IgG2, IgG3, and IgG4 antibodies were purchased from Zymed Company (San Francisco, CA) and used at a 1:500 dilution. Patterns visualized after hybridization following revelation with patients' sera were compared directly with Coomassie blue-stained blots from the same sample to determine correlation with proteins.

#### In-Gel Enzymatic Digestion and Mass Spectrometry.

The 2-D gels were stained with Coomassie blue. The proteins of interest were excised from the gels, and the Coomassie blue was removed from the gel pieces by two washes with 60% acetonitrile-200 mM ammonium bicarbonate; the gel pieces were then dried for 30 min in a vacuum centrifuge. The enzymatic digestions were performed by the addition of 100 ng of enzyme to each gel piece. The trypsin (Promega, Madison, WI) or the chymotrypsin (Boehringer, Indianapolis, IN) digestions were performed in 200 mM ammonium bicarbonate. The Glu-C (Pro-

mega) digestion was performed in 100 mM sodium phosphate buffer (pH 7.8). After enzymatic digestion overnight at 37°C, the resultant peptides were extracted twice with 50 µl of 60% acetonitrile-1% trifluoroacetic acid. After removal of acetonitrile by centrifugation in a vacuum centrifuge, the peptides were concentrated in C<sub>18</sub> pipette tips (Millipore, Bedford, MA). Analyses were performed on a Voyager-DE MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) operated in the delayed extraction mode. Peptide mixtures were analyzed using a saturated solution of cyano-4-hydroxycinnamic acid (Sigma) in acetone containing 1% trifluoroacetic acid (Sigma). Peptides were selected in the mass range of 800-4000 Da. Spectra were calibrated using calibration mixture 2 of the Sequazyme peptide mass standards kit (PerSeptive Biosystems). The search program MS-Fit, developed by the University of California at San Francisco,<sup>4</sup> was used for searches in the NCBI database. Typical search parameters were as follows: maximum allowed peptide mass error of 300 ppm, consideration of one incomplete cleavage per peptide, and pH range between 4 and 8.

**IHC.** IHC for RS protein was performed using routine formalin-fixed, paraffin-embedded sections and the Ventana ES automated IHC instrument (Ventana Medical Systems, Tucson, AZ). Rabbit polyclonal antibody was used at a 1:4000 dilution,

<sup>4</sup> <http://prospector.ucsf.edu>.

Table 2 Assignment of peptide masses to the human RS/DJ-1 sequence  
Analysis by MALDI-TOF mass spectrometry of the peptide masses followed by search in the NCBI database.

Measured mass (Da)	Matched (Da)	Delta (ppm)	Residues	Sequence
Trypsin				
875.8823	875.9826	114.5041	42–48	DPVQCSR
1159.3799	1159.2890	78.3719	90–98	EILKEQENR
1547.0638	1546.7425	207.7298	49–62	DVVICPDASLEDAK
1677.4343	1676.6554	285.6045	13–27	GAEEMETVIPVDVMR
2283.2122	2282.7201	215.5653	100–122	GLIAAICAGPTALLAHEIGFGSK
2410.9219	2410.8949	11.1956	99–122	KGLIAAICAGPTALLAHEIGFGSK
2385.8840	2835.9015	–7.3287	6–27	ALVILAKGAEEMETVIPVDVMR
2586.1042	2585.8833	85.4138	64–89	EGPYDVVVLPGGNLGAQNLSESAVK
Chymotrypsin				
983.4457	983.1567	293.9127	68–77	DVVVLPGGNL
1013.2704	1013.2734	–3.0080	1–10	ASKRALVIL <sup>a</sup>
1072.0384	1072.3168	–259.6541	102–112	IAAICAGPTAL
1146.9828	1147.2344	–219.3025	142–151	SENRVKDGDL
1186.4784	1186.2706	175.1733	154–164	TSRGPSTFEF
2942.1274	2942.5262	–135.5434	11–38	AKGAEEMETVIPVDVMRRAKIGKVTVAGL
3235.2369	3234.6441	183.2789	39–67	AGKDPVQCSRDDVVICPDASLEDAKKEGPY
Glu-C				
760.9006	760.8281	95.2907	144–149	NRVEKD
1117.1193	1117.3111	–171.6654	50–59	VVICPDASLE
1352.8936	1352.6664	168.0011	177–189	VAAQVKAPLVLDK
1375.4694	1375.6134	–104.7112	164–176	FALAIVEALNGKE
1435.4879	1435.6256	–95.9287	150–163	GLILTSRGPSTFE
1567.5786	1567.7887	–134.0037	69–84	VVVLPGGNLGAQNLSE
1598.6701	1598.8685	–124.0827	117–131	IGCGSKVTTHPLAKD
1598.8402	1598.8900	–31.1375	1–16	ASKRALVILAKGAE <sup>a</sup>
1678.6752	1678.8892	–127.4453	148–163	KDGLILTSRGPSTFE
2971.5911	2971.3955	65.8432	117–143	IGFGSKVTTHPLAKDKMMNGGHYTYSE
2972.2967	2971.8563	171.2222	60–84	DAKKEGPYDVVVLPGGNLGAQNLSE

<sup>a</sup> The peptide mass match is compatible with an Acet-N modification.

and the tissue sections were stained according to manufacturer's protocol, using the Ventana Basic DAB Detection Kit, which uses the avidin-biotin-complex method for detection of the primary antibody (21).

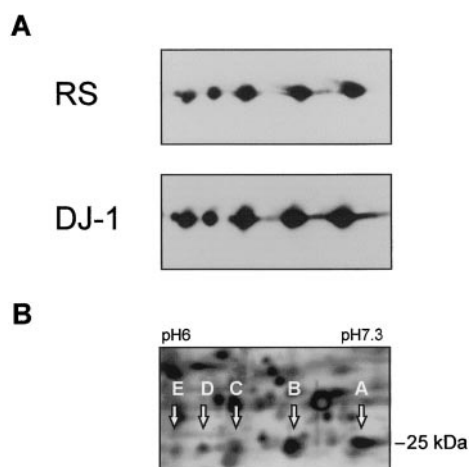
## RESULTS

**Autoantibodies to Breast Tumor Proteins in Sera from Patients with Breast Cancer.** SUM-44 proteins were separated by 2-D PAGE and transferred onto Immobilon-P PVDF membranes. Sera from 30 newly diagnosed patients with breast cancer and from 25 healthy controls were screened individually for antibodies to SUM-44 protein. Each membrane was treated with one serum sample as the primary antibody and with sheep antihuman IgG as secondary antibody. In general, several reactive protein spots were observed with most sera. Some of the reactive protein spots were observed in control sera and thus were considered to represent nonspecific reactivity. Proteins with restricted reactivity included three proteins with an estimated molecular mass of 25 kDa that showed strong reactivity with sera from four of the patients with breast cancer and none of the controls (Fig. 1 and Table 1). These three proteins showed concordant reactivity with the same sera, suggesting that given their identical molecular masses, they represented isoforms of the same protein. Sera were also tested for reactivity with proteins from heterologous tumors. Sera that were reactive against the set of three proteins also showed concordant reactivity against the same set of proteins in 2-D PAGE separations

and Western blotting of heterologous primary breast tumors (data not shown). To determine the IgG subtypes(s) of the autoantibodies, membranes obtained with SUM-44 lysates were hybridized with reactive sera and subsequently incubated with a horseradish peroxidase-conjugated mouse antihuman IgG1, IgG2, IgG3, or IgG4 antibody. Of the four IgG subtypes tested, only IgG1 reacted with this set of three protein spots (data not shown).

### Identification of the Reactive Proteins as RS/DJ-1.

The proteins of interest were extracted from the gels following 2-D PAGE and silver staining. The proteins were digested with trypsin, chymotrypsin, and Glu-C, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. The corresponding spectra were used for a protein search in the NCBI database, using the MS-FIT search program. The three protein isoforms were identified as RS (NCBI accession no. 2460318), a protein with a molecular mass of 19.89 kDa and a pI of 6.33. Altogether, the peptides obtained after the three different enzymatic digestions encompassed all of the amino acid sequence (Table 2). Identity with RS was confirmed with 2-D Western blotting using SUM-44 whole-cell extracts and an anti-RS rabbit polyclonal antibody (22). A high reactivity was observed with the set of three proteins. Reactivity was also observed with two additional low-intensity proteins (Fig. 2). It has previously been suggested that RS is identical to the oncogene DJ-1 (Refs. 22, 23; Unigene Hs. 10958). To confirm that RS and DJ-1 represent the same protein, 2-D Western blotting was performed with a

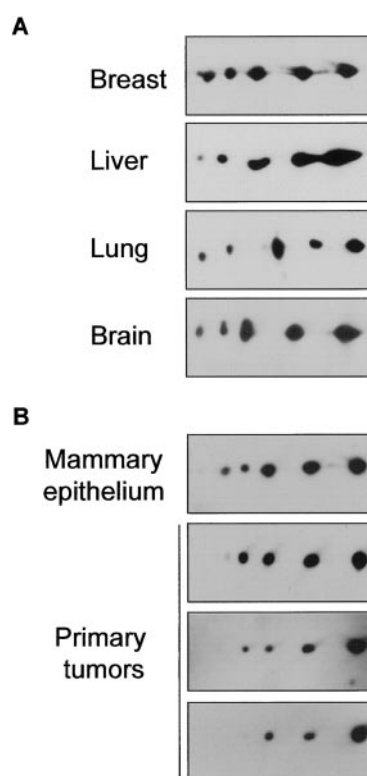


**Fig. 2** Characterization of RS by Western blot. The proteins from the breast cell line SUM-44 were separated by 2-D PAGE. **A**, the proteins were transferred to a PVDF membrane, and Western blotting was performed using the rabbit polyclonal antibody directed against RS or the mouse monoclonal antibody directed against DJ-1. **B**, the proteins from SUM-44 cells were silver stained. The five different forms of RS/DJ-1 are labeled. Only a part of the gel is shown.

mouse monoclonal anti-DJ-1 antibody. The same set of five proteins were recognized with both antibodies, confirming that RS is identical to DJ-1 (Fig. 2A). We designated these five isoforms RS/DJ-1A, B, C, D, and E with an estimated molecular mass of 25 kDa and pIs of 7.16, 6.77, 6.46, 6.27, and 6.1, respectively (Fig. 2B). The five isoforms of RS/DJ-1 exhibited the same molecular mass but different pIs, suggesting posttranslational modification(s). The different isoforms did not result from phosphorylation because they did not label with  $^{32}\text{P}$ , in contrast to the phosphorylated forms of heat shock protein 27, which migrated in the same area of the gel and were relied on as an internal control (data not shown).

**Analysis of RS/DJ-1 Expression by 2-D PAGE.** RS/DJ-1 expression was examined in a variety of tissues and tumor types by 2-D PAGE and Western blotting (Fig. 3). All RS/DJ-1 isoforms were present in different cell lines, including breast (SUM-44), liver (HuH7), lung (A549), and the glioma cell line U87 (Fig. 3A). A similar expression pattern was also observed in 15 breast tumors and in normal mammary epithelium (Fig. 3B). All isoforms were also observed in a variety of normal tissues and in gastric, esophageal, pancreatic, and brain tumors (data not shown). These results suggest that the five isoforms of RS/DJ-1 are ubiquitously expressed.

**RS/DJ-1 Autoantibodies in Sera from Patients with Different Tumor Types.** Autoantibodies against RS/DJ-1 were investigated in sera from newly diagnosed patients with different types of cancer. Autoantibodies directed against RS/DJ-1 were found in sera from 2 of 54 patients with lung cancer. None of the sera from 46 liver cancer patients exhibited autoantibodies against RS/DJ-1. To determine whether RS/DJ-1 autoantibodies were directed against an altered form of the RS/DJ-1 protein, a reactive serum from a patient with breast cancer was used for Western blotting with a lysate from normal mammary epithelium. A similar pattern of reactivity was observed

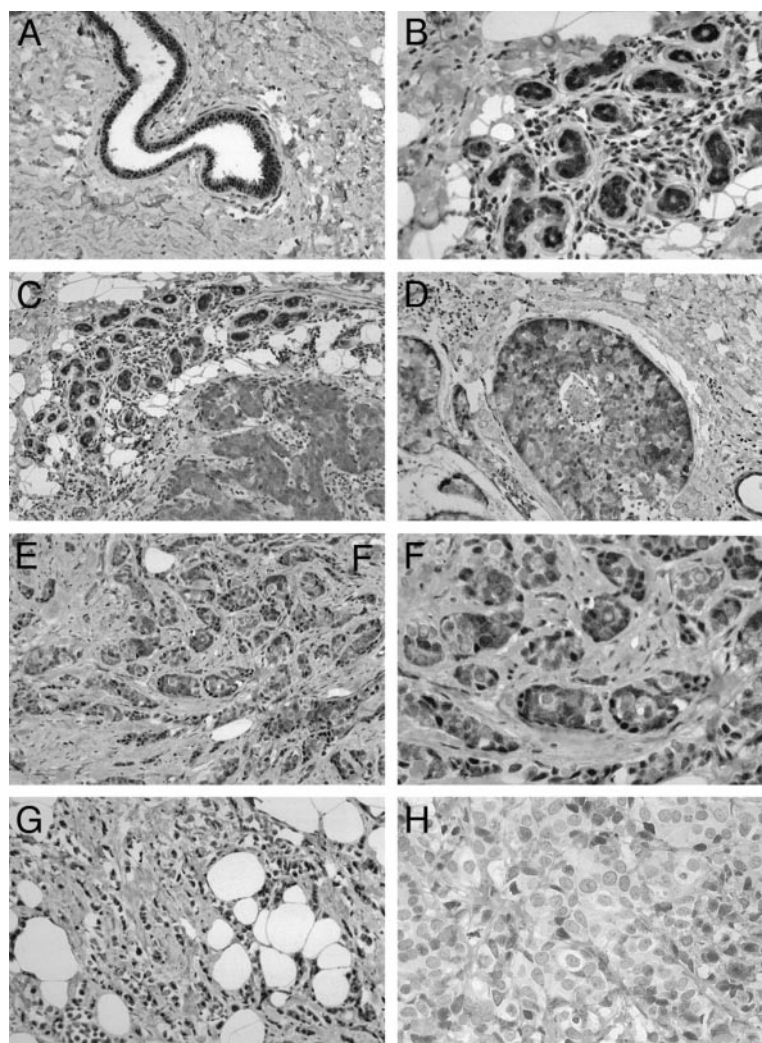


**Fig. 3** RS/DJ-1 expression analysis by Western blot. **A**, the proteins from several cell lines were separated by 2-D PAGE and transferred to a PVDF membrane. The expression of RS/DJ-1 was investigated by Western blotting using the rabbit polyclonal antibody directed against RS and whole-cell extracts from the cell lines SUM-44 (Breast), HuH7 (Liver), A549 (Lung), and U87 (Brain). **B**, RS/DJ-1 expression was investigated in normal mammary epithelium as well as in three primary breast tumors.

with tumor lysates, indicating that antigenicity was not attributable to a neoepitope associated with tumorigenesis.

**IHC Analysis of RS/DJ-1.** RS/DJ-1 expression in normal and breast tumor tissue was investigated by IHC. In the normal tissue, expression of the protein was observed in epithelium, smooth muscle, blood vessels, and nerves. All 15 (100%) invasive ductal carcinomas and 3 (100%) invasive lobular carcinomas showed some degree of RS cytoplasmic and nuclear immunoreactivity. One case of pure ductal carcinoma *in situ* and 8 of 8 (100%) of cases of ductal carcinoma *in situ* associated with invasive ductal carcinoma also showed cytoplasmic and nuclear immunoreactivity. The normal breast ductal and lobular epithelium adjacent to the carcinomas showed more intense and diffuse cytoplasmic and nuclear immunoreactivity compared with the carcinomas. In some carcinomas, there was a specific loss of nuclear immunoreactivity (Fig. 4). These results indicate a decrease in RS/DJ-1 cytoplasmic expression levels in tumor cells with loss of nuclear localization.

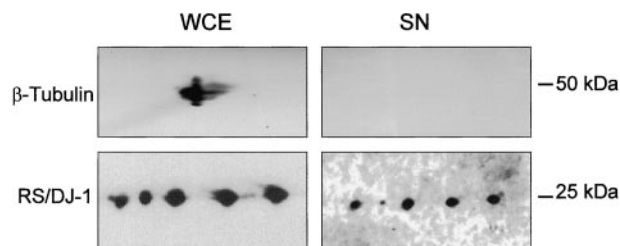
**RS/DJ-1 Is a Secreted Protein.** Given the overall diminished levels of RS/DJ-1 in breast tumor cells and the development of a humoral response to the protein in breast cancer patients, we investigated whether the protein may be secreted. SUM-44 cells were cultured as described in "Materials and Methods." Western blot experiments were performed using membranes prepared with



**Fig. 4** IHC. Sections of breast tissue containing healthy and tumor cell areas were immunostained with the rabbit polyclonal antibody against RS. The nuclei in the epithelial cells from the healthy tissue were stained with a high intensity, whereas most of nuclei in the carcinoma area were not stained. These results are representative of the observations for 20 slides.

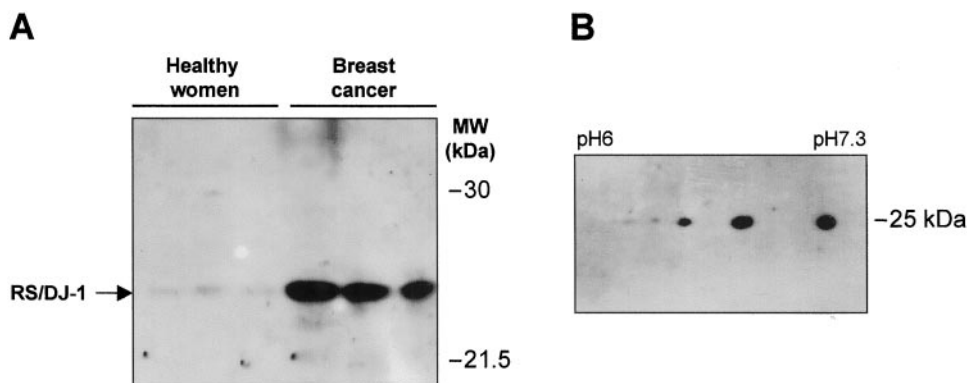
whole-cell extracts or with supernatant from SUM-44 cells. The presence of RS/DJ-1 protein in the supernatant was demonstrated by Western blotting. With anti-RS/DJ-1 antibody, spots corresponding to the molecular mass of RS/DJ-1 were observed with the supernatant, whereas no reactivity was observed in blots from the same supernatants when a tubulin- $\beta$  antibody was used as a control for cell degradation (Fig. 5). These results suggest that RS/DJ-1 protein is a secreted protein in breast tumor cells.

**Presence of RS/DJ-1 Protein in Sera from Patients with Breast Cancer.** Given the *in vitro* evidence that RS/DJ-1 may be secreted in breast tumor cells, we determined whether RS/DJ-1 protein was detectable in patient sera. Serum proteins from patients with breast cancer or from healthy individuals were separated by 1-D PAGE and then transferred onto PVDF membranes. Western blot analysis was performed using an anti-DJ-1 monoclonal antibody. A band corresponding to the molecular mass of RS/DJ-1 was observed with high intensity in sera from 37% (11 of 30) newly diagnosed breast cancer patients (Fig. 6A). None of the normal sera exhibited a comparable intensity band, although a band at the limit of detectability was present in



**Fig. 5** Occurrence of RS/DJ-1 in the secreted protein fraction of SUM-44. Proteins from whole cell extracts (WCE; left) or from supernatant of SUM-44 cells (SN; right) were separated by 2-D PAGE and transferred to a PVDF membrane. Expression of RS/DJ-1 and  $\beta$ -tubulin was determined by Western blotting experiments.

some sera. Thus, a much greater proportion of patients had detectable RS/DJ-1 antigen in circulation than detectable RS/DJ-1 antibody. All four of the patients with detectable RS/DJ-1 antibodies also had detectable antigen (Table 2). To determine



**Fig. 6** Presence of the protein RS/DJ-1 in the sera from patients with breast cancer. **A**, serum proteins from breast cancer patients or from healthy women were separated by 1-D PAGE and transferred to a PVDF membrane. Western blotting experiments were performed with the mouse monoclonal antibody against DJ-1. **B**, serum containing the protein RS/DJ-1 were used for Western blotting experiments after 2-D PAGE. The different RS/DJ-1 isoforms of the protein RS/DJ-1 are circulating.

whether a particular form of RS/DJ-1 was circulating, 2-D Western blot experiments were additionally performed with sera containing RS/DJ-1 protein. All forms of RS/DJ-1 were observed (Fig. 6B). These results suggest that RS/DJ-1 is circulating at low levels in sera from some healthy individuals and at higher levels in sera from breast cancer patients.

## DISCUSSION

We have implemented a proteomics-based approach to identify proteins that elicit a humoral response in breast cancer patients. This approach allows screening by Western-blot analysis of patient sera for antibodies that react against separated tumor cell proteins. This study was focused on a search for autoantibodies to breast tumor proteins in the pI range of 4–7.5 present in the breast cell line SUM-44. We have shown that a humoral response directed against RS, which is detectable in SUM-44 and in breast tumors, occurred in 13% of patients with breast cancer. We have demonstrated by Western blot analysis that the protein RS, which has recently been cloned (22), is identical to the protein DJ-1 (23). We have demonstrated that RS/DJ-1 protein is detected in peripheral blood at higher levels in breast cancer patients relative to healthy individuals. The elevated level of RS/DJ-1 in the serum of breast cancer patients and the lower intracellular level of the protein in the tumor cells suggest translocation of the protein from the intracellular to the extracellular environment during tumor development. There is prior evidence of a pathological translocation of RS/DJ-1 in rodents. Epididymal fluid from rats rendered infertile by oral administration of the drug ornidazole, but not from normal rats, was found to contain RS/DJ-1, which was accompanied by loss of RS/DJ-1 in epididymal sperm of infertile rats (24).

We have shown that RS/DJ-1 was secreted by the breast cancer cell line SUM-44. The release of RS/DJ-1 into the serum in patients with breast cancer could be the result of active secretion during tumor development and progression and may account for the production of autoantibodies. It cannot be ruled out that some of the circulating RS/DJ-1 protein may be the result of tumor necrosis and cell death and not necessarily antigen secretion. It should be noted that RS/DJ-1 was detected in the sera of all patients with autoantibodies. We have shown that RS/DJ-1 has five different isoforms that are ubiquitously expressed. RS has been described as a novel protein that regulates RNA-protein interaction (22). RS is a component of a 400-kDa protein complex that has been shown to be a cAMP-regulated RNA-binding protein with an affinity for

a variety of sequences with predicted stem-loop structures (22, 25). This complex consists of the RBS and RS (22). RS/DJ-1 has been shown to bind the RBS and to inhibit its RNA-binding activity (22). It has been reported that RNA-binding proteins play an important role in the control of gene expression through a variety of post-transcriptional mechanisms that control mRNA stability, localization, and translation (26). The low expression levels of RS/DJ-1 that we observed in breast tumor cells relative to the normal tissue may affect cell transformation by increasing the RNA-binding activity of the RBS.

The RS/DJ-1 gene is located on chromosome 1 at 1p36.33-p36.12, in a region that has been proposed to contain tumor suppressor gene(s) involved in breast carcinoma (27–29). On the other hand, it has been reported that RS/DJ-1 transformed mouse NIH3T3 by itself and showed a cooperative transforming activity with H-ras more than three times as strong as the activity of the ras/myc combination (23). This study has also revealed that RS/DJ-1 was not expressed in many nuclei in tumoral cells, whereas the protein was highly expressed in the nuclei of epithelial cells in healthy tissue. Interestingly, the RBS is not expressed in the nucleus (22), and RS/DJ-1 does not contain any known nuclear retrieval sequence (22, 23). In the nucleus, RS/DJ-1 may be involved in other activities unrelated to the RBS and be retained by molecular association with some other proteins, which remain to be determined.

Although RS/DJ-1 antibodies and circulating antigens were largely restricted to patients with breast cancer among the different subject groups we investigated, further studies are needed to determine the specificity of RS/DJ-1 antibodies and circulating antigens to breast cancer. For example, increased circulating levels of RS/DJ-1, with or without autoantibodies, may be affected by inflammatory changes accompanying tumorigenesis. Thus, it would be informative to determine the levels of circulating RS/DJ-1 antigen in patients with other breast lesions. In addition, the relationship between tumor burden and circulating antigen or antibody needs further clarification. Other approaches are available for the identification of antigenic proteins, notably SEREX (30). A major feature of the proteomic approach we have implemented is that tumor proteins are screened in the (modified) state in which they occur in cells. This is difficult to achieve with other screening approaches, such as SEREX, that rely on recombinant proteins that may lack antigenic and other modifications necessary for reactivity with autoantibodies. The significance of subclass restriction of RS/DJ-1

autoantibodies to IgG1 remains to be determined. Other autoantibodies to some tumor antigens, *i.e.*, anti-Yo antibodies in paraneoplastic syndrome (31) and antiannexin antibodies in lung cancer,<sup>5</sup> have been found to be restricted to IgG1. Although in our limited series the occurrence of RS/DJ-1 antigen or antibody was not limited to patients with advanced-stage disease, assessment of the potential utility of RS/DJ-1 for early diagnosis of breast cancer needs to be addressed in future studies. It is clear, however, that the proteomic approach we have implemented has the potential to identify novel proteins that may have clinical utility in cancer.

## ACKNOWLEDGMENTS

We thank Dr. Y. Hod for the generous gift of RS antibody, Dr. S. Ethier for providing the SUM-44 cell line and breast tumor samples via the tissue procurement service, and Dr. D. B. Wall for expert technical assistance in MALDI-TOF mass spectrometry.

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# Clinical Cancer Research

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François Le Naour, David E. Misek, Melissa C. Krause, et al.

*Clin Cancer Res* 2001;7:3328-3335.

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