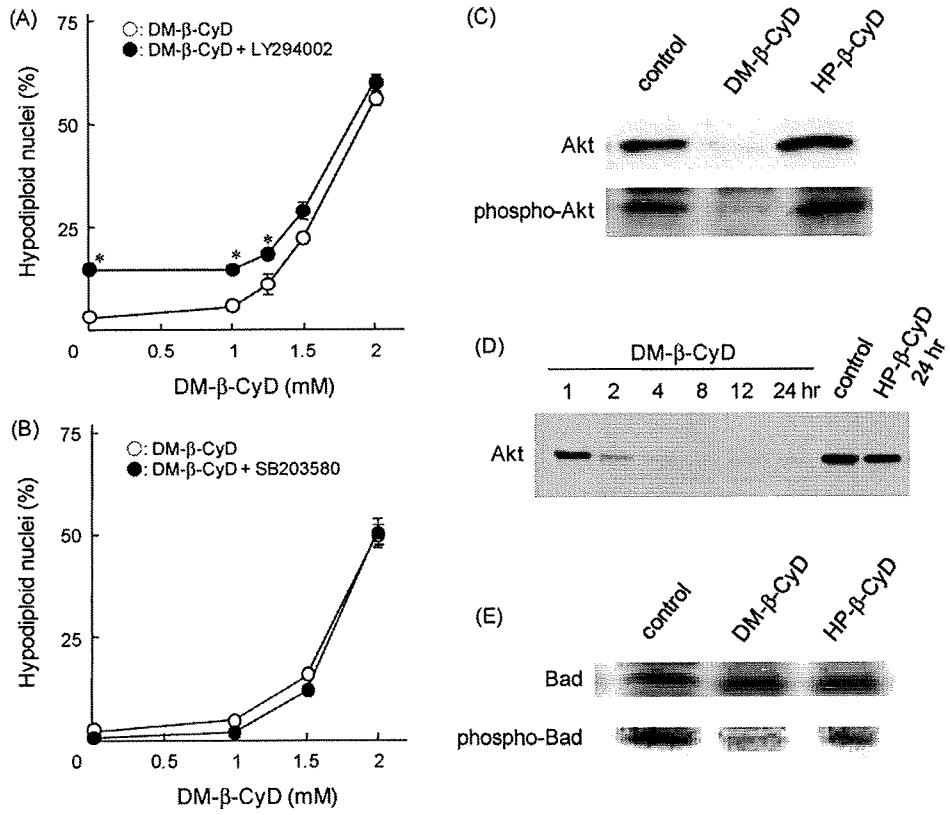

Fig. 7. Effects of PI3K inhibitor (A) and p38 MAP kinase inhibitor (B) on apoptosis induced by DM-β-CyD. Cells were treated with or without LY294002 (10 mM), a PI3K inhibitor for 4 h, or SB203580 (20 mM) for 2 h at 37 °C, and then treated with DM-β-CyD for 24 h at 37 °C. The percentage of cells showing DNA degradation was quantified by flow cytometry after attaining by PI, DM-β-CyD; DM-β-CyD + LY294002 or SB203580. Each point represents the mean ± S.E.M. of three experiments. *$p < 0.05$ *versus* DM-β-CyD. Immunoblot analysis of Akt and phospho-Akt (C and D), Bad and phospho-Bad (E), following treatment with β-CyDs. Cells were treated with 5 mM β-CyDs for 24 h or indicated time at 37 °C.

a whole, these findings strongly suggest that cell death inducted by DM-β-CyD results from apoptosis, not necrosis.

Generally, it is difficult for CyDs to induce apoptosis after uptake into cells, because CyDs have poor membrane permeability due to its aqueous properties and high molecular weight. Therefore, we hypothesized that apoptosis induced by CyDs is involved in the interaction with cell membrane components. Actually, non-ionic surfactants, such as polyethylene glycol sorbitan monolaurate (Tween 20), polyoxyethylenehydrogenated caster oil 60 (HCO-60) and Triton X-100, induced apoptosis in NR8383 cells at more than critical micelle concentrations, probably due to the solubilizing effects of cell membrane components such as cholesterol and phospholipids (data not shown). Therefore, it is highly likely that the solubilizing effects of DM-β-CyD and TM-β-CyD on membrane lipids result in apoptosis, because these methylated β-CyDs have high hemolytic activity through the solubilizing effects on cholesterol (Irie and Uekama, 1997, 1999). However, the possibility that methylated enter cells cannot be ruled out, because methylated CyDs are amphiphilic compounds. Unfortunately, we have no data regarding the membrane permeability of methylated CyDs. Elaborate study is further required for revealing the relationship between apoptotic activity and membrane permeability of methylated CyDs.

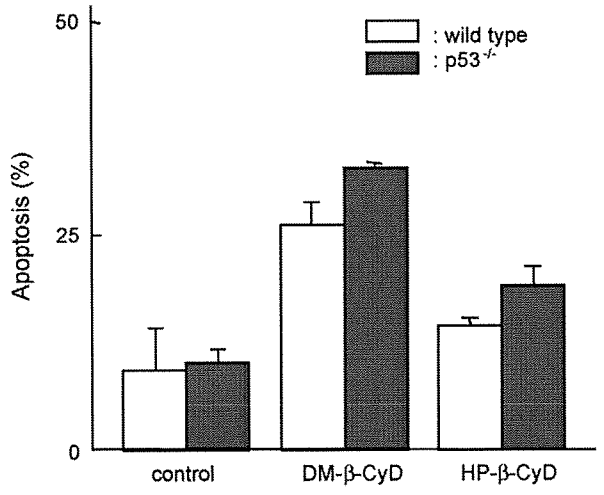

Fig. 8. Effects of β-CyDs on exposure of PS in peritoneal macrophages isolated from wild type or p53-deficient mice. Mouse peritoneal macrophages derived from wild type or $p53^{-/-}$ were treated with β-CyDs (5 mM) for 24 h, and stained with annexinV-FITC and PI, and analyzed using a flow cytometry. Each value represents the mean ± S.E.M. of three experiments.

Recently, it is reported that lipid rafts, which are lipid microdomains mainly composed of cholesterol and sphingolipids, are contributed to apoptosis via FasL/Fas and Bad, an apoptosis inducible factor of Bcl-2 family (Ayllon et al., 2002; Hueber et al., 2002; Legler et al., 2003). In addition, we previously revealed that DM-β-CyD extracted cholesterol from lipid microdomains from Caco-2 cell monolayers (Yunomae et al., 2003). Therefore, we examined whether apoptosis induced by DM-β-CyD is contributed to the extraction of membrane components from lipid rafts. Of various CyDs, M-β-CyD and TM-β-CyD mainly released cholesterol, but DM-α-CyD selectively extracted phospholipids, not cholesterol, while DM-β-CyD released both cholesterol and phospholipids from NR8383 cells (Fig. 6). Of the methylated CyDs, DM-α-CyD did not induce apoptosis even under the experimental conditions where

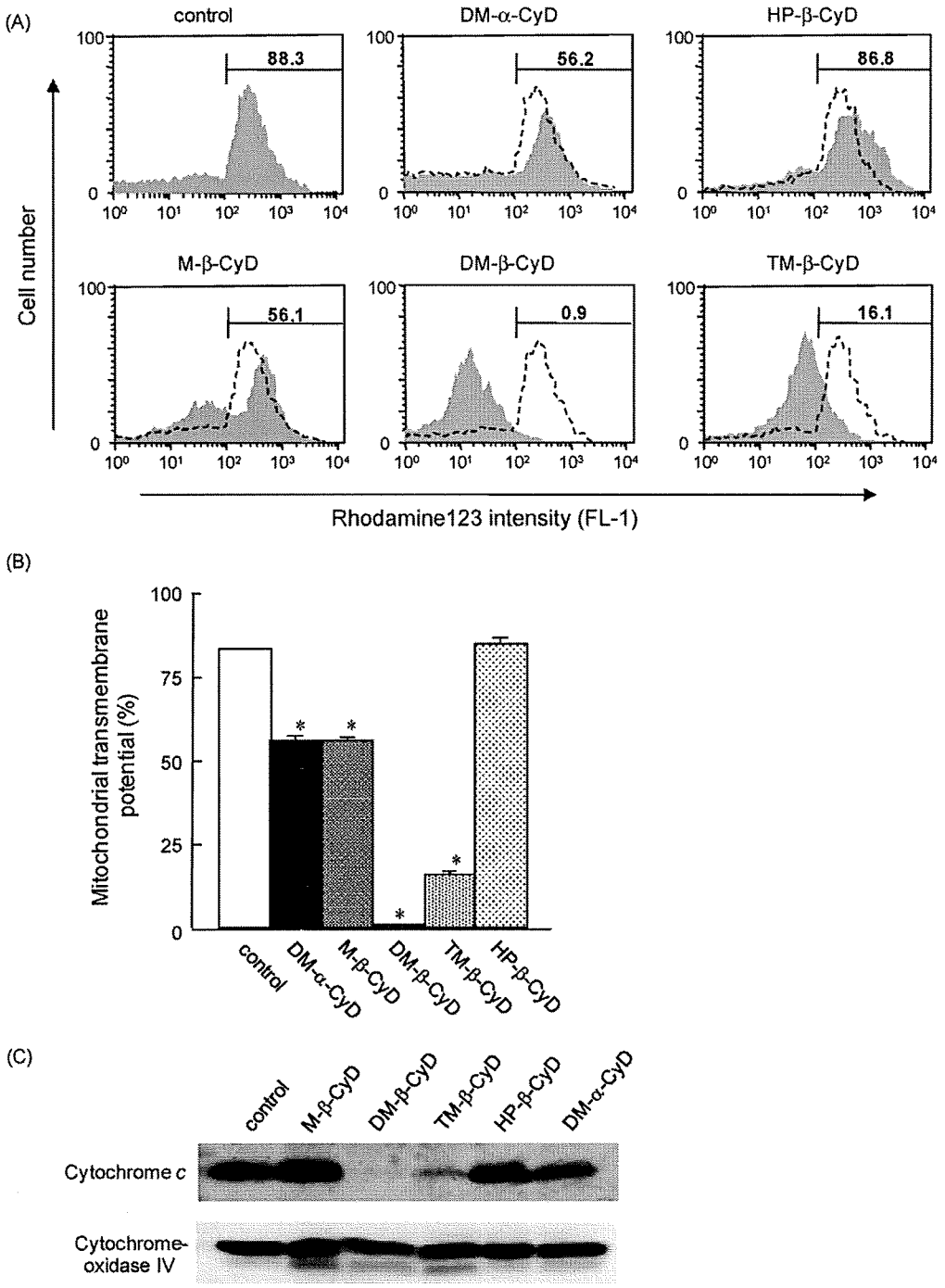

Fig. 9. (A and B) Flow cytometric analysis of mitochondrial transmembrane potential of NR8383 cells treated with CyDs. After incubation with or without CyDs (5 mM) for 24 h, cells were stained with rhodamine 123 and mitochondrial transmembrane potential was analyzed using a flow cytometry. (A) Rhodamine 123 intensity. Dotted line, untreated control and solid line, treated with CyDs. (B) Rate of mitochondrial transmembrane potential (%). Each value represents the mean ± S.E.M. of three experiments. *$p < 0.05$ *versus* control. (C) Immunoblot analysis of cytochrome *c* remaining in mitochondria following treatment of NR8383 cells with CyDs. Cells were treated with 5 mM CyDs for 24 h at 37 °C, mitochondrial fraction was isolated, and cytochrome *c* levels were assayed by Western blot. To verify the purification of mitochondrial fraction, the analysis of cytochrome oxidase IV was also carried out.

cell death was inducted (Figs. 1 and 2). Therefore, we hypothesized that cholesterol depletion in lipid rafts may have a crucial role in the induction of apoptosis by DM-β-CyD. As a result, this hypothesis may be confirmed by the results of cholesterol extraction study and of the rescue study as shown in Fig. 6. However, it remains unclear why M-β-CyD did not induce apoptosis because approximately 60% of cell death was induced under the same experimental conditions (Figs. 1A and 2). Additionally, M-β-CyD released cholesterol from NR8383 cells (Fig. 6A) and induces the same morphological change of RRBC to echinocyte as DM-β-CyD, through the extraction of cholesterol from cholesterol-rich lipid rafts (Motoyama et al., 2009). This unexpected result may be explained by the following reason: the extent of cholesterol released by M-β-CyD may be due to less than the threshold level to induce apoptosis toward NR8383 cells. Further elaborate study to address this reason should be required.

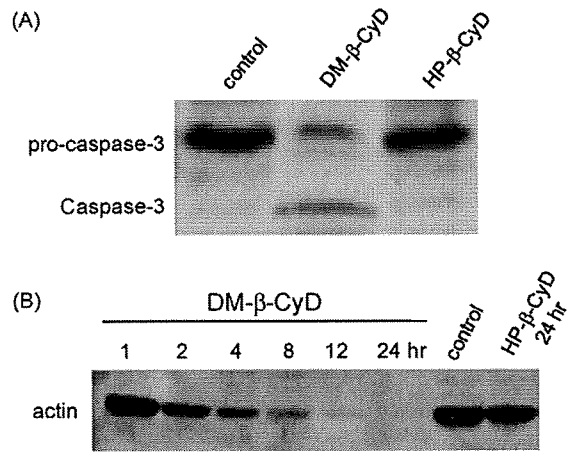

Fig. 10. Immunoblot analysis of caspase-3 (A) and actin (B) following treatment of NR8383 cells with DM-β-CyD. After incubation with 5 mM β-CyDs for 24 h at 37 °C, caspase-3 and actin levels in NR8383 cells were detected by Western blot.

PI3K activates Akt through the recruitment of Akt and PDK1 (3-phospho-inositide-dependent protein kinase-1) to lipid rafts caused by production of $PI(3,4,5)P_3$. This activated-Akt by PI3K suppresses the induction of apoptosis through the phosphorylation of Bad and caspase-9 (Cardone et al., 1998). Therefore, we investigated the effects of PI3K inhibitor (LY294002) on apoptosis induced by DM-β-CyD. The NR8383 cells pretreated with LY294002 showed more potent induction of apoptosis rather than LY294002-untreated cells under the incubation with DM-β-CyD, probably due to the synergistic effects of LY294002 and DM-β-CyD (Fig. 7A). In addition, we revealed that DM-β-CyD significantly suppressed phosphorylation of Akt and accelerated degradation of Akt in NR8383 cells (Fig. 7C and D). These inhibitory effects of DM-β-CyD on activation of Akt may be caused by impairment of recruitment of Akt to lipid rafts through the extraction of cholesterol from lipid rafts. Further study regarding the recruitment of Akt to lipid rafts after treatment with DM-β-CyD should be necessary. Furthermore, we revealed that DM-β-CyD potentially suppressed phosphorylation of Bad (Fig. 7E). Zha et al. reported that suppression of Bad phosphorylation binds with Bcl-x_L, an apoptosis inhibitory factor on mitochondrial cell membrane, resulting in induction of apoptosis (Zha et al., 1996). Therefore, these results strongly suggest the involvement of PI3K-Akt-Bad pathway in apoptosis induced by treatment with DM-β-CyD.

The regulation of p53, a tumor suppressor protein, induces apoptosis through the responses to various stresses such as DNA damage. The p53 protein is primarily regulated at the post-translational level through the ubiquitination-proteasome system, and its ubiquitination is largely induced by MDM2 (a mouse double minute 2). Recently, it is reported that RYBP (RING1 and YY1 binding protein) is a new regulator of the MDM2-p53 loop, and work as a tumor suppressor (Chen et al., 2009). It is also reported that p53 induced apoptosis through the interaction with Bak after translocation of p53 to mitochondria by stress (Leu et al., 2004). Therefore, we investigated whether p53 was involved in the induction of apoptosis by DM-β-CyD using peritoneal macrophages isolated from wild type and $p53^{-/-}$ mice. However, there was no significant difference in the amount of apoptotic cells between wild type and $p53^{-/-}$ mice after treatment with DM-β-CyD (Fig. 8). These results may suggest that the involvement of p53 such as MDM2-p53 loop and p53-Bak interaction in apoptosis induced by DM-β-CyD was only slight.

Signal transduction regarding the induction of apoptosis is largely discriminated into a mitochondrial-dependent or -independent pathway. Mitochondrion have crucial roles in signal transduction of apoptosis, because the activation of caspase family is induced through the reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) following the release of cytochrome *c* prior to apoptosis. DM-β-CyD lowered $\Delta\psi_m$ and induced the release of cytochrome *c* from mitochondria in NR8383 cells (Fig. 9). To release cytochrome *c* from mitochondria during apoptosis, the formation of VDAC (voltage-dependent anion channel) tetramer on the mitochondrial membrane plays an important role (Shimizu et al., 1999, 2001). On the other hand, Bcl-x_L regulates the release of cytochrome *c* through the suppression of VDAC channel activity (Yang et al., 1995; Zha et al., 1996). As apoptosis induction by Bad is contributed to the binding of Bad with Bcl-x_L on the mitochondrial membrane, therefore, the inhibition of phosphorylation of Bad by DM-β-CyD induced the collapse of $\Delta\psi_m$ and released cytochrome *c* from mitochondria (Figs. 7 and 9). Therefore, these results indicate that apoptosis induced by DM-β-CyD is potentially involved in the mitochondria-dependent pathway.

Cytochrome *c* released from mitochondria to cytosol activates caspase-3 through the binding with Apaf-1, ATP and pro-caspase-9 (Kroemer and Reed, 2000; Wang, 2001). In the present study, activated caspase-3 was determined in NR8383 cells treated with DM-β-CyD. In addition, degradation of actin, one of substrate of caspase-3, was observed (Fig. 10). Furthermore, apoptosis induced by DM-β-CyD in Jurkat cells was significantly suppressed by the treatment with z-VAD-fmk, a caspase inhibitor (data not shown). Collectively, these results suggest that activation of caspase-3 is important to induce apoptosis by treatment with DM-β-CyD.

DM-α-CyD may induce necrosis through depletion of sphingomyelin in sphingolipid-rich lipid rafts, because DM-α-CyD did not induce apoptosis in the present study. Actually, our preliminary studies demonstrated that DM-α-CyD released high mobility group box 1 (HMGB1) from NR8383 cells (data not shown), which may support the presumption that DM-α-CyD induces necrotic cell death, because HMGB1 released from cells is known to be a necrosis marker (Tu et al., 2009). In addition, it is well known that ceramide which is synthesized by sphingomyelinase and ceramide synthase is associated with apoptosis, and the decrease in the ceramide or sphingomyelin level in cell membranes may suppress the apoptotic pathway (Pettus et al., 2002). We previously reported that DM-α-CyD released sphingomyelin from sphingolipid-rich lipid rafts of RRBC (Motoyama et al., 2009). Thus, these lines of evidence suggest that disruption of sphingolipid-rich lipid rafts induces necrosis rather than apoptosis toward cells. Taken together, these findings may play a role in a better understanding of the involvement of cholesterol-rich lipid rafts in apoptosis, not sphigolipid-rich lipid rafts. Further elucidation of the apoptotic mechanism involved in cholesterol-rich lipid rafts, not sphingolipid-rich lipid rafts, in various cells may provide insight into the progression and extent of cell death study. Potentially, DM-β-CyD, M-β-CyD and DM-α-CyD may be useful for agents to examine the role of cholesterol-rich lipid rafts and sphingolipid-rich lipid rafts in various events in cells.

Grosse et al. (1998) found that M-β-CyD itself had excellent antitumor activity compared to doxorubicin *in vivo*. After M-β-CyD was administered intraperitoneally during 2 months to Swiss nude mice xenografted with MCF7 or A2789 cells, the antiproliferative activity of M-β-CyD was statistically higher than that of doxorubicin without loosing any body weight. Thus, M-β-CyD is likely to be more preferable to doxorubicin from the viewpoint of both pharmacological and side effects. Considering the findings of the present study, it is possible that DM-β-CyD and TM-β-CyD have the potential to be favorable antitumor agents to M-β-CyD. We are planning to examine the antitumor activity in tumor-bearing mice.

It is important to discuss the safety profiles of parent CyDs, HP-β-CyD and SBE7-β-CyD, because these CyDs are in widespread

clinical use. Parenteral administration of α-CyD and β-CyD, not γ-CyD, has been reported to cause renal toxicity, due to low aqueous solubility and the complexation ability with endogenous lipids, since kidneys is the main organ for the removal of CyDs from systemic circulation and for concentrating CyDs in the proximal convoluted tubule after glomerular filtration (Irie and Uekama, 1997). However, hemolytic activity of parent CyDs is markedly lower than that of methylated CyDs, although the mechanism of hemolysis induced by parent CyD and methylated CyDs is essentially based on the complex formation of CyDs with membrane lipid (Irie and Uekama, 1999). Under the present experimental conditions, α-CyD, β-CyD or γ-CyD did not provide cell death at the concentration of 5 mM in NR8383 cells (Fig. 2B). Thus, it is evident that parent CyDs provide less cytotoxicity than methylated CyDs, although parenteral application of α-CyD and β-CyD should require careful attention. Meanwhile, we demonstrated here that HP-β-CyD had much less cytotoxic activity than methylated CyDs (Figs. 1–10). In addition, HP-β-CyD and SBE7-β-CyD are known to have less hemolytic behavior or less toxic tubular nephritis than β-CyD, because SBE7-β-CyD and HP-β-CyD caused substantially less membrane disruption and are excreted in the urine faster and to a greater extent than β-CyD (Rajewski et al., 1995; Goule and Scott, 2005). Importantly, SBE7-β-CyD has a greatly reduced ability to solubilize cholesterol relative to HP-β-CyD. These lines of evidence suggest that parent CyDs, SBE7-β-CyD and HP-β-CyD have higher safety profile than methylated CyDs.

In conclusion, the present study may demonstrate that DM-β-CyD, not DM-α-CyD, induced apoptosis through the inhibition of the activation of PI3K-Akt-Bad pathway, resulting from cholesterol depletion in lipid rafts of cell membranes. These results will provide useful information for the involvement of various lipid rafts in cell death.

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Research Article

Detection of autoantibodies against cyclophilin A and triosephosphate isomerase in sera from breast cancer patients by proteomic analysis

Much interest is presently being shown toward identifying markers for the detection of breast cancer. To detect autoantibodies that could represent diagnostic markers for breast cancer, we comprehensively analyzed serum autoantibodies showing immunoreactivity to proteins in tumor tissues of breast cancer. Tumor tissues were obtained from 40 patients with breast cancer, along with sera from 30 other patients with breast cancer and 22 healthy donors. Proteins from tumor tissues were separated by 2-DE. After blotting onto PVDF membranes, tissue proteins were immunoblotted with sera from patients or healthy donors. By comparing each immunoblot pattern, three immunoreactive spots displayed stronger staining intensity with patient sera than with sera from healthy donors. The matched protein spots on 2-DE gels were digested and used for LC-MS/MS analysis, and identified as cyclophilin A (peptidyl-prolyl *cis-trans* isomerase A), triosephosphate isomerase and ubiquitin-conjugating enzyme E2N. Immunoblot analysis was then performed using commercially available purified proteins, confirming the specificity of anti-cyclophilin A and anti-triosephosphate isomerase antibodies in sera from patients.

Keywords:
Autoantibody / Breast cancer / Cyclophilin A (peptidyl-prolyl *cis-trans* isomerase A) / Triosephosphate isomerase / Tumor markers
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1 Introduction

Breast cancer is one of the most common cancers in women worldwide, with an incidence of 1.1 million new cases annually [1]. Early diagnosis, including mammographic screening, is a key factor in the control of breast cancer. Routine mammography, *i.e.* screening for breast cancer by radiographic examination, is currently the accepted method for early detection of breast cancer, and a recent meta-analysis of seven large-scale studies has confirmed the value of this approach as a screening tool [2]. In recent years, breast cancer mortality rates have declined as a result of earlier detection and more effective therapies [3, 4]. However, the development of new diagnostic methods to detect breast cancer in the very early stages remains important. Great interest has been shown in identifying markers for the detection of breast cancer. Autoimmunity has been shown against several proteins in breast cancer, such as p53, heat-shock protein (HSP)90, c-erbB-2/HER2/neu(HER2), mucin-related protein, and RS/DJ-1 protein [5–12]. The presence of anti-p53 autoantibodies has been observed in 15% of patients with breast cancer and is associated with poor prognosis [9, 11]. Autoantibodies against HSP90 are also reportedly associated with poor survival in breast cancer [8, 13]. The proto-oncogene, HER2, which encodes a growth factor receptor, is overexpressed in 20–30% of patients with breast cancer [14]. The presence of anti-HER2 autoantibodies has been observed in 11% of cases and correlates with overexpression of the protein in tumor tissues [15]. Elevated HER2 protein levels have been found in the serum of 29% of patients with breast cancer and have been associated with poor outcomes [16, 17]. Conversely, the presence of auti-MUC1 autoantibodies has been associated with a reduced risk of disease progression in patients with breast cancer [6]. The mucin protein MUC1, a transmembrane glycoprotein involved in cell-to-cell and

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Abbreviations: ER, estrogen receptor; **HCC,** hepatocellular carcinoma; **HSP,** heat shock protein; **PPI,** peptidyl-prolyl *cis-trans* isomerase; **TPI,** triosephosphate isomerase; **UBE2N,** ubiquitin-conjugating enzyme E2N; **WB,** Western blot

cell-to-extracellular matrix interactions, is frequently overexpressed and/or underglycosylated in malignant breast cancer [18, 19].

Serial measurements for biomarkers offer the potential to both detect recurrences preclinically and monitor the treatment of metastatic breast cancer [19]. Recently, a combination of conventional proteome analysis with serological screening (PROTEOMEX, as a combination of "proteomics" and "SEREX") was developed as an alternative approach to serological analysis of recombinant cDNA expression libraries (SEREX). This has led to the identification of various diagnostic and prognostic markers [20–23]. The present study analyzed the immunoreactivity of serum antibodies to tissue proteins in patients with breast cancer using PROTEOMEX analyses to detect autoantibodies that could represent diagnostic markers for breast cancer.

2 Materials and methods

2.1 Tumor tissue samples

Breast cancer tissues were obtained from 40 patients diagnosed with primary breast cancer who had undergone surgical mastectomy or mammary gland partial excision in the Department of Surgery II at Yamaguchi University Hospital (from November 2000 to July 2005). Tissues were stored at −80°C immediately after resection. Written informed consent was obtained from all patients prior to surgery. None of the patients had received any preoperative therapies such as chemo- or hormonotherapy. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi University School of Medicine. Histological diagnosis of breast cancer was made on formalin-fixed, paraffin-embedded sections according to General Rules for Clinical and Pathological Recording of Breast Cancer (15th Edition) in Japan.

Clinical characteristics of the breast cancer patients whose cancer tissues were used for 2-DE are presented in Table 1.

2.2 Serum samples

A total of 52 serum samples were used, with 30 samples randomly selected from patients with breast cancer, who had undergone surgical mastectomy or mammary gland partial excision in the Department of Surgery II at Yamaguchi University Hospital (from April 2003 to November 2004) and 22 samples obtained from healthy donors as controls. All serum samples were stored at −80°C until use. The patients with breast cancer, whose sera were used for this study, had not been diagnosed as any autoimmune disease. Clinical characteristics of the breast cancer patients whose sera were used are presented in Table 1. Sera from patients with pancreatic cancer, hepatocellular carcinoma (HCC), esophageal cancer or fibroadenoma of breast were used.

Table 1. Clinical characteristics of the breast cancer patients whose cancer tissues were used for 2-DE (a) and whose sera were used (b)

Factor	No.	Factor	No.	Factor	No.
(a)					
Age (y-o)		*Lymph node metastasis*		*ER*	
≤35	1	Positive	22	Positive	30
35< ≤50	10	Negative	18	Negative	8
50<	29	*Stage*[a)]		Unknown	2
Menopause		I	10	*PgR*	
Pre	12	II	22	Positive	24
After	26	III	7	Negative	14
Unknown	2	IV	1	Unknown	2
Tumor size		*Pathology*		*HER2*	
≤1c	1	Scirrhous	21	0	9
1< ≤2	12	Solid	9	1+	3
2< ≤3	15	Papillo	9	2+	9
3<	12	Others	1	3+	7
				Not measured	12
(b)					
Age (y-o)		*Lymph node metastasis*		*ER*	
≤35	1	Positive	12	Positive	21
35< ≤50	7	Negative	18	Negative	7
50<	22	*Stage*[a)]		Unknown	2
Menopause		I	12	*PgR*	
Pre	8	II	14	Positive	19
After	22	III	2	Negative	9
		IV	2	Unknown	2
Tumor size		*Pathology*		*HER2*	
≤1c	2	Scirrhous	15	0	4
1< ≤2	14	Solid	4	1+	3
2< ≤3	6	Papillo	8	2+	8
3<	8	Others	3	3+	11
				Not measured	4

a) Stage: General Rules for Clinical and Pathological Recording of Breast Cancer (15th Edn.) in Japan.

2.3 Sample preparation

Breast cancer tissues were suspended and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% NP-40). Suspensions were incubated for approximately 2 h at 4°C and centrifuged at 15 000 × *g* for 30 min at 4°C, then the supernatants were stored at −80°C until use [24]. Protein concentrations were determined using Lowry's method [25].

2.4 2-DE

The sample for 2-DE (200 μg) was composed of equal amounts of protein (5 μg) from cancer tissues of 40 patients. For one set, 200 μg of protein was used for 2-D immunoblotting (blotting gels). For the other set, 300 μg of protein was used for CBB staining (staining gels). For each set of

samples, the first-dimensional IEF was performed on 7-cm, immobilized, pH 3–10, linear gradient strips (BIO-RAD Laboratories, Hercules, CA) at 20°C and 50 mA/strip. The strips were rehydrated with 125 μL of rehydration solution including samples (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 0.56% 2-mercaptoethanol and 0.5% IPG buffer) for 14 h. IEF was run in three steps at 20°C: 500 V for 1 h; 1000 V for 1 h and 8000 V for 4 h. Voltage increases were performed in a gradient [26].

In the second dimension, SDS-PAGE was performed using 85 mm (*W*) × 60 mm (*L*) × 1 mm (*T*) slab-type gels (15%*T*, 2.67%*C*) at 15 mA/plate. After electrophoresis, blotting gels were used for 2-D immunoblotting, and staining gels were stained using a See Pico CBB Kit (Benebiosis, Seoul, Korea) for 24 h.

2.5 2-D immunoblot analysis

For blotting gels, fractionated proteins were transferred electrophoretically onto PVDF membrane (Immobilon-P; Milli-pore, Bedford, MA); then the membranes were blocked for 1 h at 4°C with TBS containing 5% skimmed milk. Membranes were subsequently incubated for 2 or 3 days at 4°C with sera (1:50 dilution), washed four times with TBS containing 0.05% Tween 20, and incubated for 1 h at 4°C with horseradish peroxidase-conjugated secondary antibody (1:2000, 62-8320; ZYMED Laboratories, South San Francisco, CA). Sera from 20 patients with breast cancer and 20 healthy donors were used and compared in this experiment. The reaction was visualized with a chemifluorescence reagent (ECL Plus; GE Healthcare, Buckinghamshire, UK) [27].

Patterns visualized after hybridization following revelation with patient sera were compared directly with See Pico CBB-stained blots from the same sample to determine correlations with proteins.

After we did Western blot (WB) and 2-DE at the same time, we picked up the spots that became marks. In addition, we pulled lines and we printed images of WB and two DE gel and made spots a mark and compared it visually. We put paper of the 2-D image, which we printed on paper of the WB image and lighted it up, and put it together and confirmed spots.

2.6 Reprobing

For blotting with other serum samples, stripping was performed in stripping buffer (0.7% 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7). After blocking for 1 h with TBS containing 5% skimmed milk, membranes were incubated with other serum samples.

2.7 In-gel digestion

For staining gels, CBB dye was removed by three rinses in 60% methanol, 50 mM ammonium bicarbonate and 5 mM DTT for 15 min and twice in 50% ACN, 50 mM ammonium bicarbonate and 5 mM DTT for 10 min. Gel pieces were dehydrated three times in 100% ACN for 30 min, then rehydrated in an in-gel digestion reagent containing 10 μg/mL sequencing-grade modified trypsin (Promega, Madison, WI) in 30% ACN, 50 mM ammonium bicarbonate and 5 mM DTT. In-gel digestions were performed overnight at 30°C. Samples were rinsed in 30% ACN, 50 mM ammonium bicarbonate and 5 mM DTT for 2 h and lyophilized overnight at −30°C [28].

2.8 Amino acid sequencing by LC-MS/MS

Lyophilized samples were dissolved in 20 μL of 0.1% formic acid and centrifuged at 15 000 × *g* for 5 min. Peptide sequencing of identified protein spots was performed with an Agilent 1100 series LC/MSD Trap XCT (Agilent Technologies, Santa Clara, CA). Twenty-five microliters of each sample was applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm; Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: Solvent A, 0.1% formic acid; Solvent B, CH_3CN in 0.1% formic acid; column flow, 0.3 μL/min; primary flow, 300 μL/min; gradient, 0–5 min 2% B, 60 min 60% B; and stop time, 60 min [29].

2.9 2-D immunoblot analysis of cyclophilin A and triosephosphate isomerase

Fifty micrograms of proteins from breast cancer tissues were used to confirm the presence of cyclophilin A and triosephosphate isomerase (TPI) proteins. After electrophoresis, immunoblot analysis was performed as described in Section 2.5. The primary antibodies were a rabbit anti-cyclophilin A antibody (1:1000 dilution, upstate cell signaling solutions, Lake Placid, NY) and a goat anti-TPI antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated for 1 h at 4°C with a horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (1: 5000 dilution; Cappel, ICN Biomedicals, Aurora, OH).

2.10 1-D immunoblot analysis for confirmation of autoantibodies specificity

Three commercially available recombinant proteins were used to confirm the specificity of autoantibodies: cyclophilin A (#se105; BIOMOL, Plymouth Meeting, PA); ubiquitin-conjugating enzyme E2N (UBE2N) (#ag0292; Proteintech Group, Chicago, IL); and TPI (#H00007167-P01; Abnova, Taipei, Taiwan). Each SDS-PAGE used 1 μg of cyclophilin A, 1 μg of UBE2N and 100 ng of TPI. Sample lysates were mixed with 5 μL of 1 × SDS sample buffer.

Samples were denatured at 95°C for 5 min and separated on 10, 12.5 or 15% SDS-PAGE gels. Electrophoresis

was performed at 15 mA/gel. After electrophoresis, immunoblot analysis was performed as described in Section 2.5. Sera from 28 patients with breast cancer and 21 healthy donors were used and compared in this experiment.

Furthermore, sera from three patients with pancreatic cancer, three patients with HCC, eight patients with esophageal cancer or eight patients with fibroadenoma of breast were added. The presence of anti-cyclophilin A autoantibody was compared with estrogen receptor (ER) status because correlation between endocrine responsiveness and cyclophilin A has been reported [30]. The presence of anti-TPI autoantibody was compared with HER2 status because correlation between HER2 and TPI has been reported [31]. ER status and HER2 status were checked by immunohistochemistry.

ER was judged to be positive in more than 10% based on a calculated percentage according to international criteria. ER was calculated as a percentage of whether an immunoreactive found thing occupies several percentages of the whole tumor without a relation to the staining intensity. HER2 was judged to be positive, which satisfied the following according to international criteria. There is a cell presenting HER2 positive in neoplastic cell in a specimen organization of more than 10%. Staining characteristics show localized intensity on a membrane of the neoplastic cell.

2.11 Immunoblot analysis of cyclophilin A and TPI in breast cancerous and non-cancerous tissues

Breast cancerous tissues and paired non-cancerous tissues were analyzed by Western blot analysis. The expression levels of target proteins were normalized with the level of actin in the same blot, and each tumorous tissue was compared with its paired non-cancerous tissues. Breast cancerous tissues and paired non-cancerous tissues from more 21 patients with breast cancer were added, and this experiment was performed. The expression of cyclophilin A was compared with ER status. The expression of TPI was compared with HER2 status.

2.12 RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitorogen, CA) following the manufacturer's instructions. Breast cancerous tissues and paired non-cancerous tissues were homogenized in TRIzol reagent (1 mL/100 mg tissue) for full lysis. After the homogenate was left for 5 min at room temperature, chloroform (0.2 mL/mL TRIzol reagent) was added and it was mixed completely. Then, after 3 min at room temperature, the homogenate was subjected to centrifugation at 12 000 rpm for 15 min at 4°C. Next, the upper phase (500 μL) was transferred into a new tube, incubated with isopropanol (0.5 mL/mL TRIzol reagent) for 10 min at room temperature, and centrifuged at 12 000 rpm for 10 min at 4°C. The RNA pellet was washed with 75% ethanol (1 mL/TRIzol reagent) by centrifugation at 12 000 rpm for 5 min at 4°C. After drying for 10 min at room temperature, the RNA pellet was resolved in 0.1 mL of autoclaved MilliQ water, and the RNA was quantified by measuring the absorbance with a UV spectrophotometer at a wavelength of 260 nm.

Total RNA (1 μg) was incubated at 65°C for 5 min and was then cooled down on ice.

Thereafter, 1 μg of total RNA was reversibly transcribed to cDNA using five buffers, 0.1 M DTT, dNTPs (Sigma-Aldrich, St. Lois, MO), 1 mg/mL oligo dT primer (invitrogen), and 200 U of reverse transcriptase in a final volume of 20 μL. The suspension was then incubated at 37°C for 1 h, 65°C for 5 min, and then placed on ice. The PCR reaction was performed at 94°C for 2 min, 94°C for 15 s, 55°C for 30 s and 68°C for 1 min for 35 cycles. The primers used here are: cyclopilin A, forward: 5′-CTCCTTTGAGCTGTTTGCAGACAAGG-3′ and reverse: 5′-CACCACAT6CCATCCAACCACTC-3′ (325 bp). The PCR products were analyzed on a 3% agarose gel and stained with 0.4 mg/mL ethidium bromide.

2.13 Pull-down experiment for antibodies with recombinant proteins of cyclophilin A and TPI

One microgram of recombinant cyclophilin A was incubated in sera (1% milk: 1:50 dilution) for 2 days. One hundred nanogram of recombinant TPI was incubated in sera (1% milk: 1:50 dilution) for 2 days. After centrifugation (2000 × *g*, 10 min), the supernatant was used as autoantibody pulled down sera.

Recombinant proteins were denatured at 95°C for 5 min and separated on 15% SDS-PAGE gels. Electrophoresis was performed at 15 mA/gel. After electrophoresis, immunoblot analysis was performed as described in Section 2.5.

2.14 Correlation between autoantibodies directed against both proteins and other known tumor markers

Known tumor markers such as CEA and CA15-3 in the breast cancer patient sera, which were used for this study, were investigated. The tumor markers mainly used in this hospital were CEA, CA15-3, BCA225 and NCC-ST439. Each tumor marker were measured by enzyme-linked immunosorbent assay method. The value of those preoperative tumor markers was examined and compared with the presence of autoantibodies against cyclophilin A and TPI.

2.15 Statistics

Data were analyzed by using Pearson's χ^2 test. The χ^2 test was performed to determine the differences between groups. $p < 0.05$ was considered as statistically significant.

3 Results

3.1 Differential expression of spots recognized by autoantibodies in sera from breast cancer patients and healthy donors

Immunoreactivity of serum autoantibodies to breast cancer tissue proteins was assessed by 2-D immunoblotting. Several spots were visualized on the membranes (Fig. 1). Sera from 20 patients with breast cancer and 20 healthy donors were used and compared in this experiment.

Spot patterns on each membrane were compared visually. Recognition of no spot at all was judged as a negative. A spot was judged as positive even if only faint.

Three immunoreactive spots showed positive with sera from breast cancer patients compared with control sera (Fig. 1). Protein spot 1 was recognized by 90% (18/20) of sera from breast cancer patients and 35% (7/20) of control

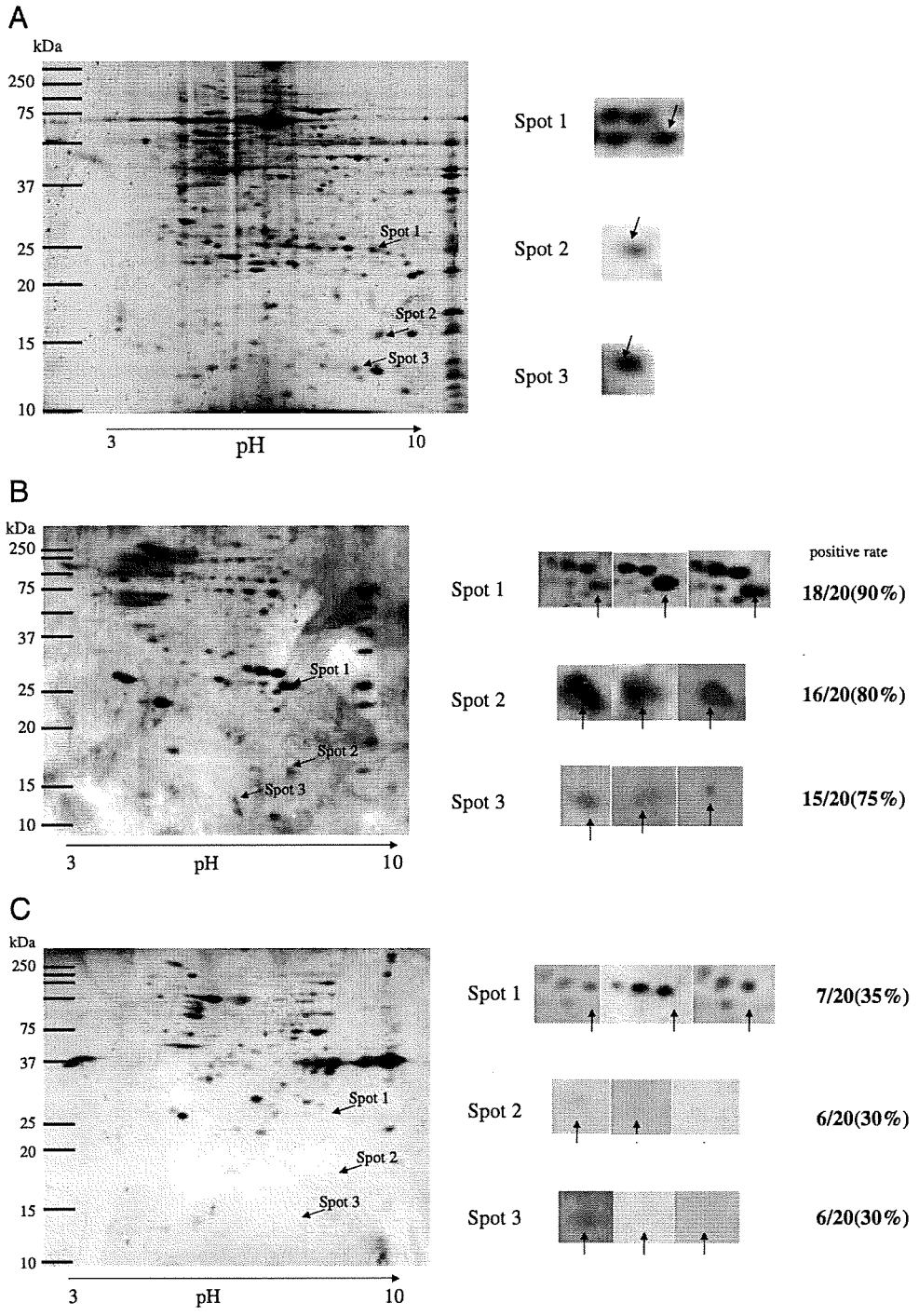

Figure 1. 2-D immunoblotting patterns. (A) See Pico CBB staining of proteins from breast cancer tissues separated by 2-DE. (B) 2-D immunoblotting patterns of protein spots from breast cancer tissues reacting with serum sample from breast cancer patients. (C) 2-D immunoblotting patterns of protein spots from breast cancer tissues reacting with serum sample from healthy donors. Three immunoreactive spots with sera from breast cancer patients showed increased intensity compared with control sera. Patterns visualized after hybridization following revelation with patient sera were compared directly with See Pico CBB-stained blots from the same sample to determine correlations with proteins. Sera from 20 patients with breast cancer and 20 healthy donors were used and compared.

sera ($p = 0.0002$). Protein spot 2 was recognized by 80% (16/20) of sera from breast cancer patients and 30% (6/20) of control sera ($p = 0.0036$). Protein spot 3 was recognized by 75% (15/20) of sera from breast cancer patients and 30% (6/20) of control sera ($p = 0.010$).

3.2 Identification of three immunoreactive proteins

To identify these three immunoreactive proteins, spots on the See Pico CBB-stained gel corresponding to the reactive spots were digested and used for LC-MS/MS analysis. Each sample provided good spectra of amino acid sequences, resulting in the identification of cyclophilin A, UBE2N and TPI. LC-MS/MS results are shown in Table 2 and Fig. 2.

3.3 2-D Western blotting with specific anti-cyclophilin A and anti-TPI antibodies

From the 2-D Western blotting with specific anti-cyclophilin A and TPI antibodies, the spots were confirmed as the presence of cyclophilin A and TPI proteins (Fig. 3). Spot patterns on each membrane were compared visually. Although there were some non-specific protein spots, we could identity cyclophilin A and TPI in the same position as Fig. 1B.

3.4 Confirmation of autoantibody specificity

Immunoblot analysis using commercially available recombinant proteins corresponding to the three identified proteins was performed and the specificity of each autoantibody was examined. Spot patterns on each membrane were compared visually. When no band could be recognized at all, the result was judged as negative. Bands were considered positive even if only faint. To examine specificity of anti-cyclophilin A autoantibody and anti-TPI autoantibody, sera from 28 patients with breast cancer and 21 healthy donors were used and compared in this experiment. Because no difference was seen in bands between sera from16 patients with breast cancer and 12 healthy donors, we discontinued to examine the specificity of anti-UBE2N autoantibody in this experiment.

Furthermore, sera from three patients with pancreatic cancer, two patients with HCC, three patients with esophageal cancer or three patients with fibroadenoma of breast were used to examine specificity of anti-cyclophilin A autoantibody and compared in this experiment. Sera from three patients with pancreatic cancer, three patients with HCC, eight patients with esophageal cancer or eight patients with fibroadenoma of breast were used to examine specificity of anti-TPI autoantibody and compared in this experiment.

Incidence of immunoreaction of serum autoantibodies against UBE2N did not differ between sera from patients and controls (Fig. 4A). Incidence of immunoreaction of serum autoantibodies against cyclophilin A was significantly positive in sera from breast cancer patients compared with controls ($p = 0.006$) (Fig. 4A). Incidence of immunoreaction of serum autoantibodies against TPI was significantly positive in sera from breast cancer patients compared with control sera ($p = 0.0004$) (Fig. 4A). Incidence of immunoreaction of serum autoantibodies against cyclophilin A showed no difference in sera from ER-positive breast cancer patients compared with ER-negative breast cancer patients (Table 4). Incidence of immunoreaction of serum autoantibodies against TPI showed no difference in sera from HER2-positive breast cancer patients compared with HER2-negative breast cancer patients (Table 4).

Immunoreaction of serum autoantibodies against cyclophilin A was negative in sera from fibroadenoma of breast, pancreatic cancer, HCC or esophageal cancer patients (Fig. 4B). Immunoreaction of serum autoantibodies against TPI was positive in sera from pancreatic cancer or HCC patients (Fig. 4B). Immunoreaction of serum autoantibodies against TPI was significantly positive in sera from esophageal cancer patients compared with control sera ($p = 0.027$) (Fig. 4B). Immunoreaction of serum autoantibodies against TPI was significantly positive in sera from breast cancer patients compared with sera from fibroadenoma of breast patients ($p = 0.023$) (Fig. 4B).

3.5 Expression of cyclophilin A and TPI in breast cancer tissues

Breast cancerous tissues and paired non-cancerous tissues from more 21 patients with breast cancer were used in this experiment. The expression of cyclophilin A was significantly increased in breast cancerous tissues (71%) (Table 3 and (Fig. 5). The expression of cyclophilin A significantly increased in ER-positive breast cancerous tissues in comparison with paired non-cancerous tissues ($p = 0.0055$) (Table 4). The expression of TPI was increased in breast cancerous

Table 2. Result of LC-MS/MS for the each spot

Spot	Protein name	Accession no.	p*I*	M_r	MS/MS search score	Sequence coverage (%)	Recurrence of autoantibody (%)		*p*-Value
							Cancer sera	Control sera	
1	TPI	P60174	6.45	26669	126.71	43	18/20 (90%)	7/20 (35%)	$p = 0.0002$
2	Cyclophilin A	P62937	768.68	18012	11202.02	57	16/20 (80%)	6/20 (30%)	$p = 0.0036$
3	UBE2N	P61088	6.13	17137	53.68	34	15/20 (75%)	6/20 (30%)	$p = 0.010$

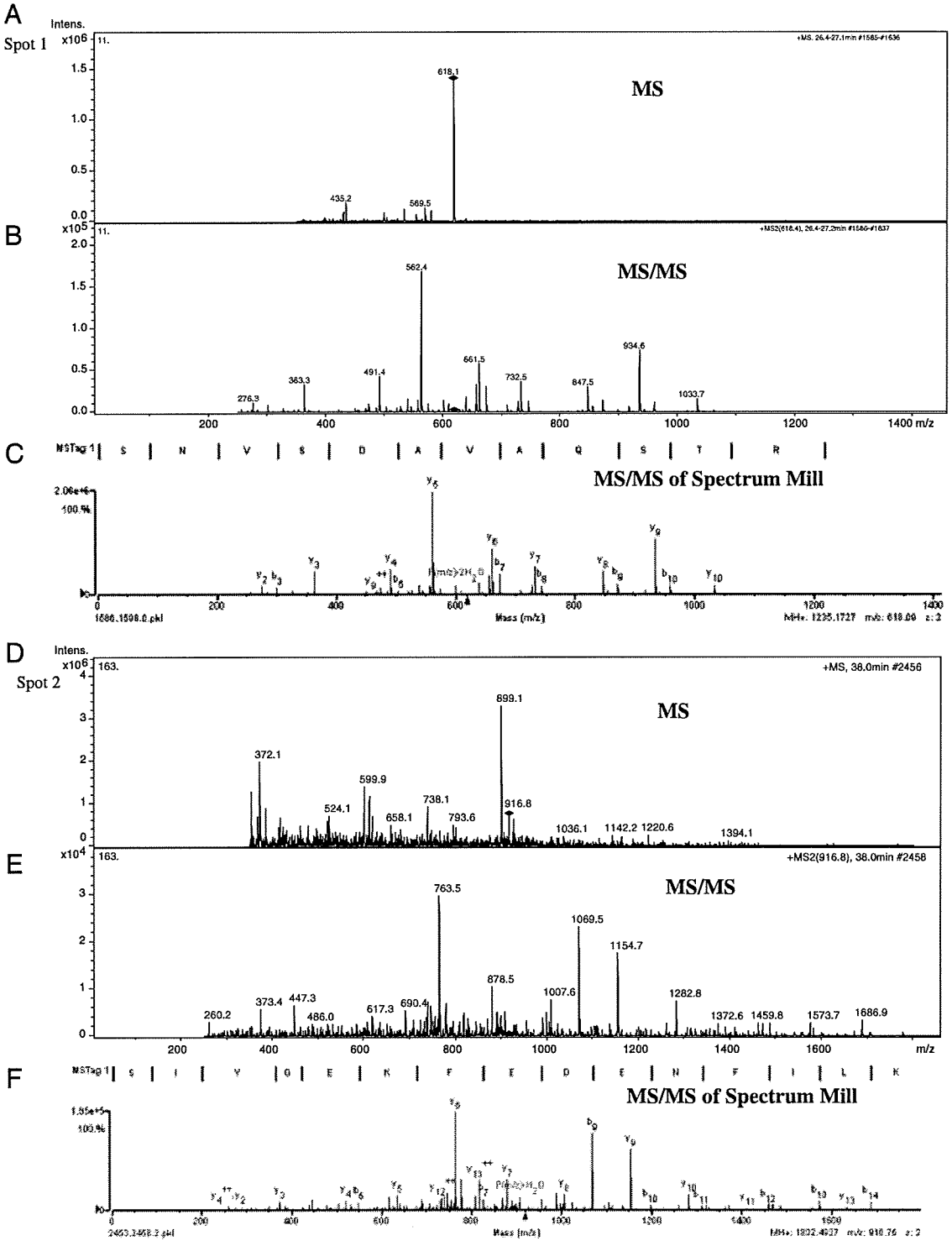

Figure 2. MS and MS/MS spectra of trypsin-digested spots of spots 1, 2 and 3. (A) LC-MS spectra of tryptic digests of spot 1; TPI; precursor ion *m/z* is 618.1. (B) and (C) LC-MS/MS spectrum of a precursor ion with *m/z* 618.1 marked by a lozenge in (A). (D) LC-MS spectra of tryptic digests of spot 2; cyclophilin A; precursor ion *m/z* is 916.8. (E) and (F) LC-MS/MS spectrum of a precursor ion with *m/z* 916.8 marked by a lozenge in (D). (G) LC-MS spectra of tryptic digests of UBE2N; precursor ion *m/z* is 602.3. (H) and (I) LC-MS/MS spectrum of a precursor ion with *m/z* 602.3 marked by a lozenge in (G).

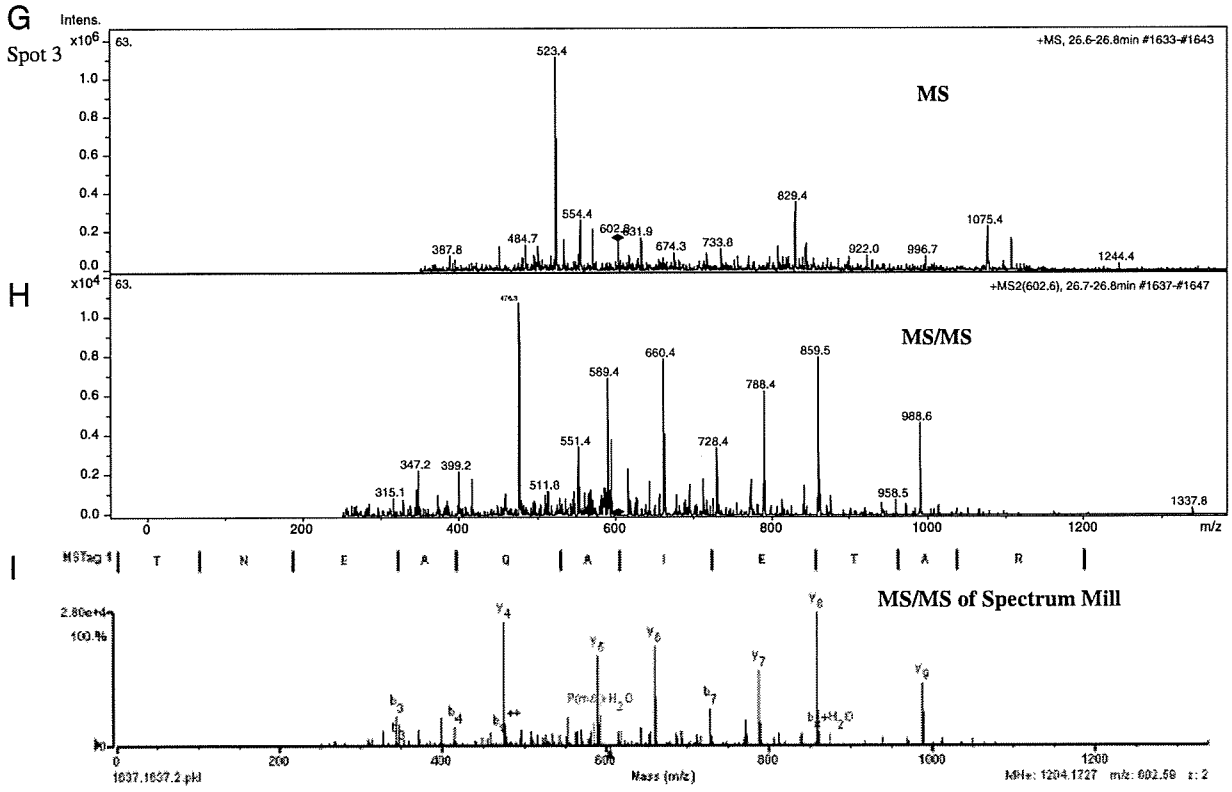

Figure 2. Continued

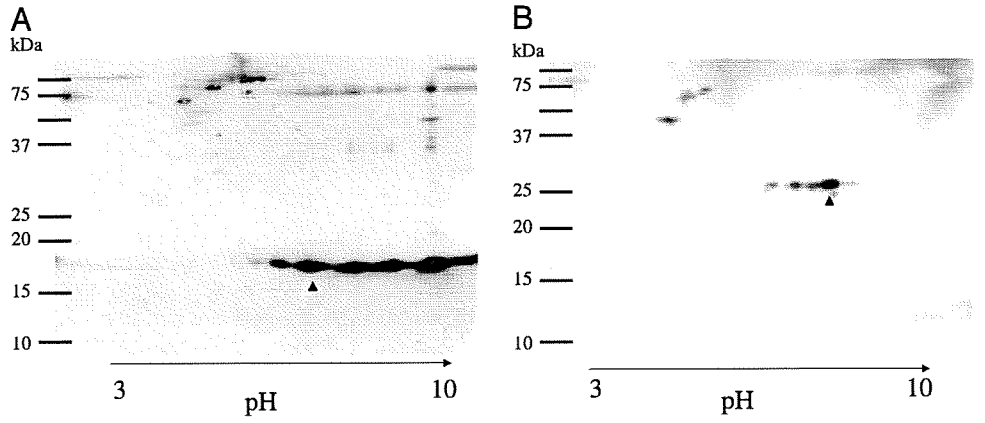

Figure 3. 2-D immunoblot analysis of cyclophilin A (A) and TPI (B). For the 2-D immunoblot analysis, 50 μg protein was applied. From the 2-D Western blotting with specific anti-cyclophilin A and TPI antibodies, the spots were confirmed as cyclophilin A and TPI proteins.

tissues (47%) (Table 3 and Fig. 5). The expression of TPI tended to increase in HER2-positive breast cancer (Table 3).

3.6 Analysis of cyclophilin A mRNA expression by RT-PCR

To examine the possibility that the reactivity of breast cancer sera is due to an elevated expression of cyclophilin A mRNA, we performed semi-quantitative RT-PCR techniques on three breast cancerous tissues and paired non-cancerous tissues.

We used two different genes, RS9 and TBP89, as internal controls and calculated the ratio between the cyclophilin A gene expression level and each internal control to normalize data. We compared normalized ratios between breast cancerous tissues and paired non-cancerous tissues. The level of cyclophilin A mRNA expression in breast cancer tumors was significantly higher.

Western blotting confirmed increased protein expression of cyclophilin A. To elucidate whether the expression of cyclophilin A mRNA, semi-quantitative RT-PCR techniques were used. Breast cancerous tissues and paired

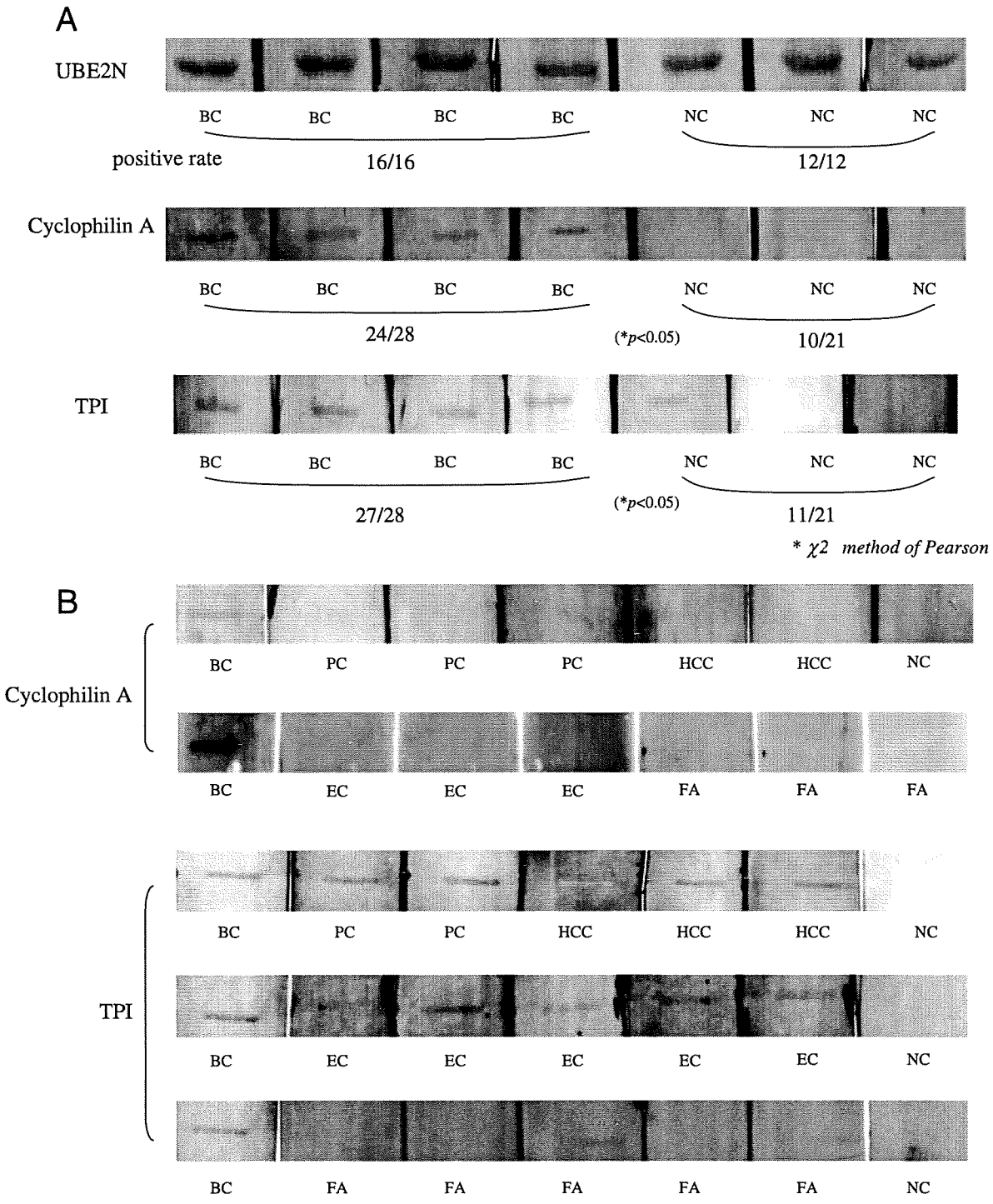

Figure 4. Confirmation of autoantibody specificity. Immunoblot analysis using commercially available recombinant proteins corresponding to the three identified proteins was performed and the specificity of each autoantibody was examined. Spot patterns on each membrane were compared visually. In sera from breast cancer patients and controls, incidence of immunoreactivity of autoantibodies to each purified protein was assessed. Sera from 28 patients with breast cancer and 21 healthy donors were used for anti-cyclophilin A autoantibody and anti-TPI autoantibody. Sera from16 patients with breast cancer and 12 healthy donors were used for anti-UBE2N autoantibody. Sera from three patients with pancreatic cancer, two patients with HCC, three patients with esophageal cancer or three patients with fibroadenoma of breast were used for anti-cyclophilin A autoantibody. Sera from three patients with pancreatic cancer, three patients with HCC, eight patients with esophageal cancer or eight patients with fibroadenoma of breast were used for anti-TPI autoantibody. BC, sera from breast cancer; NC, sera from healthy donors; PC, sera from pancreatic cancer; HCC, sera from hepatocellular carcinoma; EC, sera from esophageal cancer; FA, sera from fibroadenoma of breast.

Table 3. Result of cyclophilin A and TPI expression in breast cancer

Patient	ER	Cyclophilin A	PgR	HER2	TPI	Pathology
1	+	Increase	+	–	Increase	Solid
2	+	Increase	+	–	Increase	Solid
3	+	Increase	+	–	No difference	Scirrhous
4	+	Increase	+	+	Increase	Papillo
5	+	Increase	+	+	Increase	Scirrhous
6	+	Increase	+	–	Increase	Papillo
7	–	No difference	–	+	Increase	Solid
8	+	Increase	+	–	No difference	Scirrhous
9	+	Increase	+	–	No difference	Scirrhous
10	+	Increase	–	+	Increase	Scirrhous
11	+	Increase	+	–	No difference	Scirrhous
12	–	No difference	–	+	No difference	Scihrrhous
13	–	No difference	–	+	Increase	Scirrhous
14	+	No difference	+	+	Increase	Papillo
15	–	Increase	+	+	No difference	Papillo
16	–	No difference	–	Unknown	No difference	Scirrhous
17	+	Increase	+	–	No difference	Scirrhous
18	–	Increase	–	Unknown	No difference	Solid
19	+	Increase	+	Unknown	No difference	Solid
20	–	No difference	+	–	No difference	Solid
21	+	Increase	+	–	Increase	Solid

3.8 Correlation between autoantibodies directed against both proteins and other known tumor markers

The tumor markers of CEA, CA15-3, BCA225 and NCC-ST439 in the breast cancer patient sera were investigated.

The tumor marker of CEA was positive in only one of 30 breast cancer patients. The patient was a case of stage III where a lot of lymph node metastasis was positive. Other tumor markers were negative in all 30 breast cancer patients. As for the case in which CEA was positive, the presence of serum autoantibodies against cyclophilin A and TPI were positive (data not shown).

4 Discussion

We have implemented a proteomics-based approach to identify biomarkers eliciting a humoral immune response in breast cancer patients. The search protocol used has already been established in searches for cancer examination markers using existing autoantibodies in patient sera [32–35]. This approach was combined with Western blotting to screen autoantibodies in sera from cancer patients that react with tumor cell proteins separated by 2-DE.

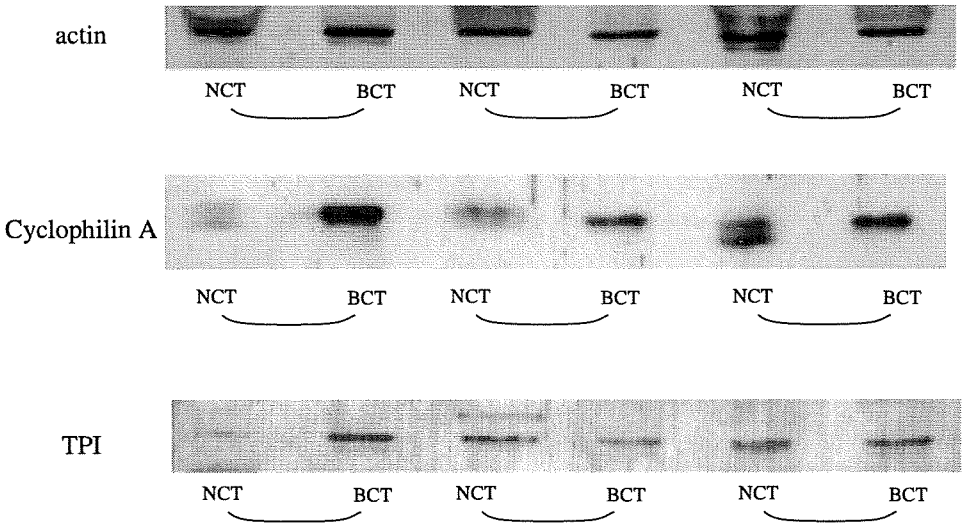

Figure 5. 1-D immunoblot analysis of cyclophilin A and TPI. Breast cancerous tissues and paired non-cancerous tissues from 21 patients with breast cancer were used in this experiment. The expression of cyclophilin A was significantly increased in breast cancerous tissues (71%). The expression of TPI was increased in breast cancerous tissues (47%). BCT, breast cancerous tissues; NCT, non-cancerous tissues.

non-cancerous tissues from three patients with breast cancer were used in this experiment. The expression of mRNA increased in breast cancerous tissues (Fig. 6).

3.7 Pull-down experiment for antibodies with recombinant cyclophilin A and TPI

Immunoblotting with only sera from breast cancer patients showed the strongest band. The band was the faintest after the pull-down of sera from breast cancer patients with 1 μg or 100 ng of recombinant protein, respectively (Fig. 7).

In this PROTEOMEX study, we identified the presence of antibodies against cyclophilin A, UBE2N and TPI in sera from breast cancer patients.

Cyclophilin A belongs to a group of proteins that display peptidyl-prolyl *cis-trans* isomerase (PPI) activity [36], and was originally identified as an intracellular receptor for cyclosporin A [37]. The immunosuppressive activity of cyclosporin A is thought to be mediated by the engagement of calcineurin by the cyclosporin A-cyclophilin A complex [38], an observation supported by the finding that cyclophilin A knockout mice are resistant to immunosuppression by

Table 4. Comparison between anti-cyclophilin A antibody and ER (a), anti TPI antibody and ER (b), expression of cyclophilin A and ER (*$p<0.05$) (c) expression of TPI and HER2

	Anti-cyclopilin A antibody positive	Anti-cyclopilin A antibody negative	
(a)			
ER positive	19	2	21
ER negative	5	2	7
	24	4	28
(b)	Anti-TPI antibody positive	Anti-TPI antibody negative	
HER2 positive	11	0	11
HER2 negative	15	0	15
Unknown	1	1	2
	27	1	28
(c)	Cyclopilin A increased	Cyclopilin A no difference	
ER positive	13	1	14
ER negative	2	5	7
	15	6	21
(d)	TPI increased	TPI no difference	
HER2 positive	6	2	8
HER2 negative	4	6	10
Unknown	0	3	3
	10	11	21

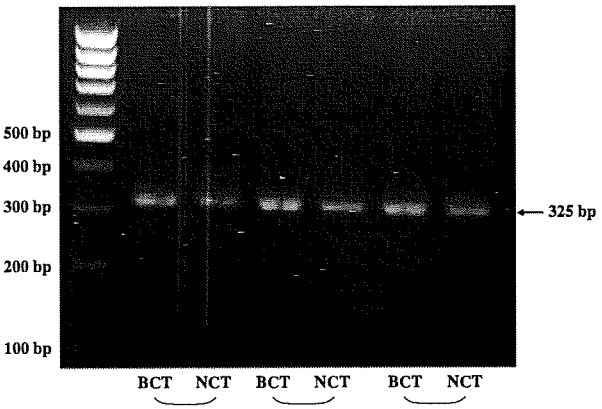

Figure 6. Semiquantitative RT-PCR analysis for cyclophilin A. Breast cancerous tissues and paired non-cancerous tissues from three patients with breast cancer were used in this experiment. The expression of mRNA increased in breast cancerous tissues. PCR was carried out for 35 cycles. BCT, breast cancerous tissues; NCT, non-cancerous tissues.

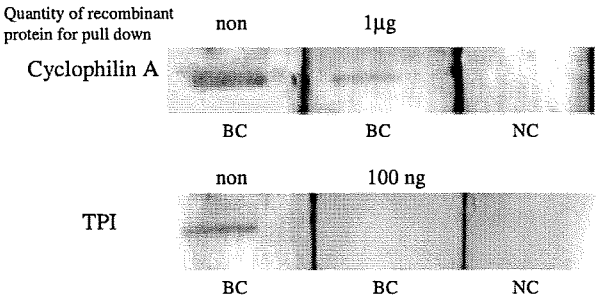

Figure 7. Pull-down experiment for antibodies with recombinant protein of cyclophilin A and TPI. BC, sera from breast cancer; NC, sera from healthy donors.

cyclosporin A [39]. As a PPI, cyclophilin A has been found to assist in protein folding *in vivo* [40]. Several lines of research, however, have revealed that cyclophilin A and other PPI may function as molecular signaling switches [41]. Cyclophilin A was secreted from cells in response to inflammatory stimuli [42]. This secreted cyclophilin A displays multiple functions in chemotaxis and cell signaling cascades through the CD147 cellular receptor. CD147 is a transmembrane receptor protein involved in neuronal function, inflammation and tumor invasion, and cyclophilin A has been shown to be a secreted growth factor induced by oxidative stress, stimulating the ERK1/2 pathway and cell proliferation in vascular smooth muscle cells [43]. The ERK1/2 pathway is thought to be stimulated predominantly by growth factors and plays an important role in cell growth and differentiation.

Additional data also suggest that cyclophilin A may contribute to the pathology of human malignancy. Cyclophilin A is overexpressed in a large number of lung [44], pancreatic [45] and oral squamous cancer cells and tissues [46]. In this study, cyclophilin A was overexpressed in breast cancer tissues. And expression of anti-cyclophilin antibody that has been reported for one of the breast tumor proteins were specifically recognized by sera from early-stage breast cancer patients [47]. Significantly, in a case cohort of 25 000 women who had

received cyclosporin A as therapy for renal and cardiac allografts, a reduction in the incidence of breast cancer of up to 50% in the cyclosporin A-treated group was noted during a ten-year follow-up period. Indeed, in their first year of therapy the cyclosporin A-treated cohort showed a 90% reduction in the incidence of breast cancer [48]. Zheng *et al.* reported that cyclophilin A, which is implicated in the regulation of protein conformation, is necessary for the prolactin-induced activation of Jak2 and the progression of human breast cancer [49]. The important role of the hormone prolactin in the development and progression of breast cancer has been supported by epidemiologic studies and transgenic model studies [50–52]. Choi *et al* have reported that overexpression of cyclophilin A in cancer cells renders resistance to hypoxia- and cisplatin-induced cell death [53]. Expression of cyclophilin B, which is a PPI family, is associated with malignant progression and regulation of genes implicated in the pathogenesis of breast cancer [54]. It was reported that the enhanced expression of cyclophilin B in malignant breast epithelium may contribute to the pathogenesis of this disease through its regulation of the expression of hormone receptors and gene products that are involved in cell proliferation and motility. The expression of cyclophilin A significantly increased in ER-positive breast cancerous tissues in comparison with paired non-cancerous tissues in this study, too. These data suggest that Cyclophilin A may play an important role in tumorigenesis and serve as a target for PPI inhibitors in the oncologic setting.

TPI is a ubiquitously expressed enzyme that catalyzes the interconversion between dihydroxyacetone phosphate and glycerol dehyde-3-phosphate in the energy metabolism pathway. TPI is ubiquitously distributed in the cytoplasm of all tissues, and tissues with high glycolytic activity generally contain high levels of TPI [55, 56]. In HER2-positive breast cancer, the increased energy requirement for cell proliferation could be partly supported by the up-regulation of the glycolytic enzymes, such as TPI [31]. It was recognized in breast cancer that enhanced glycolytic metabolism will provide more energy (ATP) for cell proliferation and growth and will increase the concentration of metabolites such as lactate and pyruvate to regulate hypoxia-inducible gene expression, including that of vascular endothelial growth factor. The significant difference was not recognized in this study, but the expression of TPI tended to increase in HER2-positive breast cancer. Antibody against TPI has been reported for one of the breast tumor proteins, which was specifically recognized by sera from early-stage breast cancer patients [47]. Antibodies against TPI have also been reported as autoantibodies in squamous cell carcinoma of lung and hepatoma [57, 58].

Ubiquitin is a common small protein comprising 76 amino acids. Ubiquitin is involved in a multienzyme system consisting of activating enzyme (E1)/conjugating enzyme (E2)/ligase (E3) (an ubiquitin system). An ubiquitin-conjugating enzyme receives ubiquitin activated by E1 as a high-energy thioester articulation. Furthermore, the ubiquitination converts the nature (duration of life and intracellular localization) of the target protein by creating a covalent bond in the target protein [59, 60].

Recently, Hudelist *et al.* reported that various proteins are overexpressed in breast cancer tissues [61], and Takashima *et al.* reported that specific autoantibodies increased in the sera of HCC patients [22]. Desmetz *et al.*, Lu *et al.*, Hamrita *et al.* and Chapman *et al.* reported on autoantibodies such as HSP60, p53, Mn-SOD, and c-myc in serum from patients with early breast cancer [47, 62–64].

Some reports have indicated that autoantibodies are produced predominantly in sera and are detectable by a highly sensitive chemiluminescence reaction, whereas the proteins themselves are not detected in sera [22]. Although the role of such circulating antibodies is unknown, substantial evidence indicates an immune response to cancer, as shown by the identification of autoantibodies against intercellular or surface antigens in sera [65]. Diversity in the autoantibodies produced in breast cancer may be a consequence of the wide variety of overexpressed or mutated proteins involved in repeated cycles of necrosis and regeneration in tumor development [66]. The pathological significance of the expression of these antibodies remains to be elucidated. Autoantibodies are frequently found in the sera of patients with cancer, but have generally been thought to be non-specific and of uncertain significance [67]. However, the recognition of specific antibodies is useful, as the immune response against tumor proteins occurs in the early stage of tumor progression when the tumor may not be detectable on imaging [65]. Generally, the immunogenicity of autoantibody in malignant tumor may depend on the level of expression, posttranslational modification or other types of processing of a protein, the extent of which may vary among tumors of a similar type. It is speculated that autoantibody may be produced so that expression of cyclophilin A increases from the results of Western blotting and RT-PCR in this study. The two antibodies detected in this study, anti-cyclophilin A antibody and anti-TPI antibody could be candidates for specific antibodies in breast cancer and diagnostic markers for breast cancer. Furthermore, anti-TPI antibody could be candidates for specific antibodies such as esophageal cancer, HCC and pancreatic cancer and a candidate of a diagnosis mark. However, diagnosis of breast cancer using autoantibody alone against cyclophilin A and TPI remains difficult from a clinical perspective. It is important to validate the markers in the future using samples from newly diagnosed breast cancer patients. We support the concept noted by recent publications in which a combination of autoantibody markers are used for cancer examination [62].

We thus expect the development of a simultaneous test for the detection of autoantibody and homologous antigen.

The authors have declared no conflict of interest.

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A HUPO test sample study reveals common problems in mass spectrometry–based proteomics

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We performed a test sample study to try to identify errors leading to irreproducibility, including incompleteness of peptide sampling, in liquid chromatography–mass spectrometry–based proteomics. We distributed an equimolar test sample, comprising 20 highly purified recombinant human proteins, to 27 laboratories. Each protein contained one or more unique tryptic peptides of 1,250 Da to test for ion selection and sampling in the mass spectrometer. Of the 27 labs, members of only 7 labs initially reported all 20 proteins correctly, and members of only 1 lab reported all tryptic peptides of 1,250 Da. Centralized analysis of the raw data, however, revealed that all 20 proteins and most of the 1,250 Da peptides had been detected in all 27 labs. Our centralized analysis determined missed identifications (false negatives), environmental contamination, database matching and curation of protein identifications as sources of problems. Improved search engines and databases are needed for mass spectrometry–based proteomics.

Liquid chromatography–mass spectrometry (LC-MS) has become the most popular technique for proteomics analysis. In this strategy, proteins of a sample are typically separated by PAGE and then digested with trypsin. After extraction from the gel, peptides are separated by liquid chromatography and upon elution are ionized via electrospray into the mass spectrometer for characterization by mass analysis. The mass spectrometer subsequently selects peptides for fragmentation to yield mass values that are then used to identify the peptide and the corresponding protein by searching sequence databases. This technique, termed tandem mass spectrometry (MS), is repeated to continuously select ionized peptides from the liquid chromatography column. Depending on protein abundance and complexity, the mass spectrometer type and its setup, up to about 15,000 peptides and up to about 4,000 proteins can be identified in a single experiment[1].

Despite the high mass accuracy of modern mass spectrometers, the general perception of the reliability of MS-based proteomics is that it is low. Previous test sample studies have demonstrated that there is both a lack of reproducibility between different laboratories as well as a general inability to identify purified proteins in samples of low complexity[2] (http://www.abrf.org/ResearchGroups/ProteomicsStandardsResearchGroup/EPosters/ABRFsPRGStudy2006poster.pdf). This is in part due to the stochastic nature of peptide sampling by the mass spectrometer and the inherent bias toward peptides of higher concentrations, which also confounds the statistical challenges and pitfalls associated with MS-based analyses, particularly when samples are rich in protein complexity. Protein solubilization, protein separation, protease digestion, peptide separation and peptide selection, all involve steps and protocols that vary greatly among labs, and different commercially available tandem mass spectrometers have different mass accuracies and different rates of peptide selection for fragmentation. The use of different search engines to decode tandem mass spectra and match them to databases of theoretical tryptic peptides is also a source of variability[3], because of differences in the search engines themselves as well as different false discovery rates[4,5]. Furthermore, the matching of high-quality tandem mass spectra to different databases may lead to irreproducibility as protein databases vary greatly in terms of their curation, completeness and comprehensiveness[6–8]. Despite variability in instruments, search engines and databases, the high mass accuracy of modern mass spectrometers[9] should assure a 100% success rate of protein identification for those tryptic peptides that readily ionize and for which high-quality tandem mass spectra can be obtained.

Prior work in analytical chemistry and genomics[10–14] has demonstrated the benefits of standardized test sample efforts for testing the reproducibility of technology platforms. To address the question of reproducibility in LC-MS–based proteomics[15], the Human Proteome Organization (HUPO) created a test sample working group to carry out a controlled study involving 27 different labs. We produced a test sample made up of 20 human proteins of high purity and at equimolar ratios. To test for any potential stochastic bottleneck as a consequence of current data-dependent acquisition methods[16], all 20 proteins were selected to contain at least one unique tryptic peptide of 1,250 ± 5 Da each with a different amino acid sequence. The primary task given to members

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