Antibody-based proteomics for esophageal cancer: Identification of proteins in the nuclear factor-κB pathway and mitotic checkpoint

Norihisa Uemura,[1,3,4] Yukihiro Nakanishi,[2] Hoichi Kato,[3] Masato Nagino,[4] Setsuo Hirohashi[1] and Tadashi Kondo[1,5]

[1]Proteome Bioinformatics Project, National Cancer Center Research Institute; [2]Pathology Division, National Cancer Center Research Institute, Tokyo 104-0045; [3]Division of Esophageal Surgery, National Cancer Center Hospital, Tokyo 104-0045; [4]Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

To identify the molecular background of esophageal cancer, we conducted a proteomics study using an antibody microarray consisting of 725 antibodies and surgical specimens from three cases. The microarray analysis identified 24 proteins with aberrant expression in esophageal cancer compared with the corresponding normal mucosa. The overexpression of 14 of the 24 proteins was validated by western blotting analysis of the same samples. These 14 proteins were examined by immunohistochemistry, in which nine proteins showed consistent results with those obtained by western blotting. Among the nine proteins, seven were localized in tumor cells, and two in infiltrating cells. The former included proteins associated with mitotic checkpoint control and the nuclear factor (NF)-κB pathway. Although mitotic checkpoint gene products (budding uninhibited by benzidazoles 1 homolog beta (BubR1) and mitotic arrest deficient-like 1 (Mad2)) have previously been reported to be involved in esophageal cancer, the association of NF-κB-activating kinase, caspase 10, and activator protein-1 with esophageal cancer has not been previously reported. These proteins play a key role in the NF-κB pathway, and NF-κB is a signal transduction factor that has emerged as an important modulator of altered gene programs and malignant phenotype in the development of cancer. The association of these proteins with esophageal cancer may indicate that mitotic checkpoint gene products and NF-κB play an important part in the carcinogenesis of esophageal cancer. (*Cancer Sci* 2009; 100: 1612–1622)

Esophageal cancer is the eighth most common cancer[1] and the sixth leading cause of cancer death worldwide.[2] Despite the use of modern surgical techniques in combination with radiotherapy and chemotherapy, early recurrence is common and the overall 5-year survival rate remains below 40%.[3–5] ESCC develops through a multistep process from dysplasia through carcinoma *in situ* to invasive carcinoma, and the acquisition of molecular alterations tightly corresponds to the dysplasia–carcinoma sequence.[6] However, the mechanisms of esophageal cancer progression remain largely obscure. The characterization of molecular alterations inherently linked to ESCC development and an in-depth understanding of the molecular mechanisms underlying carcinogenesis and growth control may therefore provide information relevant to early tumor detection, refined prognosis, and development of novel targeted therapeutics.

As the proteome is a functional translation of the genome, directly regulating cancer phenotypes, its study may further our understanding of esophageal cancer. Esophageal cancer research has made significant progress due to proteomics studies on two fronts to date. First, tissue proteomics have identified numerous intracellular proteins that had not been previously reported to be implicated in esophageal cancer. For instance, the use of two-dimensional polyacrylamide gel electrophoresis followed by mass spectrometry and database search led to the identification of the aberrant expression of proteins correlating with certain clinicopathological parameters in esophageal cancer.[7] Second, the use of proteomics tools has led to the discovery of a novel candidate plasma biomarker for early diagnosis.[8] This is important as the majority of esophageal cancer cases has advanced locally or has developed distant metastases by the time of diagnosis, rendering the cancer surgically inoperable,[9,10] and the existing plasma tumor markers (e.g. squamous cell carcinoma-related antigen (SCC), cinoembryonic antigen (CEA), and cytokeratin-19 fragments (CYFRA21-1)) have obvious limitations in terms of sensitivity and specificity in detecting patients with localized and resectable esophageal cancer.[11]

The major proteomics tools such as two-dimensional gel electrophoresis and mass spectrometry essentially have two major drawbacks. First, these techniques tend to uncover the proteins in the order of their expression level; the proteins with higher expression levels are more frequently observed than proteins with low abundance such as transcription factors, receptors, and growth factors. Second, the present approaches do not allow the systematic profiling of protein expression. The proteins are currently identified when their physical characteristics, such as the molecular weight and charge, fit the conditions of the proteomic methods, and such protein characteristics do not necessarily correlate with protein functions. As a consequence, pathway comprehensive approaches such as those identifying all proteins in certain molecular pathways cannot be carried out using the present proteomics tools. To partially address these drawbacks, multidimensional separation by liquid chromatography and gel electrophoresis were used to reduce sample complexity and increase the number of observable proteins. However, further improvements are still required to overcome the limitations of cancer proteomics.

The recently developed antibody-based proteomics may address the aforementioned limitations of cancer proteomics. A large-scale library of antibodies with high specificity and sensitivity may be used to detect the proteins with low expression levels, and thus enable pathway comprehensive analysis. For instance, a large-scale immunoblotting analysis with 900 well-characterized antibodies resulted in the identification of 102 deregulated proteins in pancreatic cancer cells.[12] An antibody proteomics project was launched to detect all proteins encoded by the human genome.[13] In addition to the characterization of antibodies already developed, the development of techniques for hundreds or thousands of antibodies will be the challenge in antibody-based proteomics.

In this paper, we examined the proteins with aberrant expression levels in esophageal cancer tissues and normal tissues using an antibody microarray. The antibody microarray is commercially

[5]To whom correspondence should be addressed. E-mail: takondo@ncc.go.jp

available and enables comprehensive high-throughput studies. We validated the antibody microarray study results by western blotting and immunohistochemistry. Through the comparison between the normal mucosa and tumor tissues, we discussed the reproducibility of the antibody microarray results, the degree of inconsistency between the different methods, and the functional contribution of the identified members of the NF-κB pathway to esophageal cancer progression.

Materials and Methods

Patients. Tumor tissues (1T, 2T and 3T) and their adjacent normal mucosal tissues (1N, 2N and 3N) were collected from three cases of ESCC, which were surgically resected in 2007 and 2008 at the National Cancer Center Hospital. The resected tissues were snap frozen in liquid nitrogen and stored at –80°C until use. All cases were newly diagnosed as squamous cell carcinoma of the esophagus (ESCC). The patients did not receive anticancer treatment prior to surgery. All patients were male and in the seventh decade of their lives, and all tumors were located in the lower thoracic segment of the esophagus and were classified as T3N1 according to the International Union against Cancer TNM classification.[14] Only one case with distant lymph node metastasis was classified as M1.[14] This study was approved by the ethics committee of the National Cancer Center and written informed consent was obtained from the patients.

Antibody microarrays. The Panorama Antibody Microarray XPRESS Profilter725, XP725 was purchased from Sigma-Aldrich (St Louis, MO, USA). Seven hundred and twenty-five antibodies were spotted on each glass slide in 32 subarrays, each containing duplicate spots for 23 antibodies, as well as duplicate positive control spots for Cy3 and Cy5 (monoclonal antibodies labeled with Cy3 and Cy5), and duplicate negative controls, resulting in 1536 spots per slide. These antibodies represent families of proteins known to be involved in a variety of different biological pathways. Detailed information on each antibody can be obtained in the antibody specific datasheet found on the Sigma-Aldrich website at http://www.sigma.com/arrays.

Protein extraction. Frozen samples were crushed to powder with a CryoPress (Microtech Nichion, Shizuoka, Japan) under cooling with liquid nitrogen. The frozen powder was then treated with urea lysis buffer (7 mol/L urea, 2 mol/L thiourea, 3% CHAPS, and 1% Triton X-100). After centrifugation at 17 400 g for 10 min, the supernatant was used as the source of cellular proteins for protein expression studies. The protein concentrations ranged between 3.46 and 8.45 mg/mL.

Protein labeling and microarray assays. Briefly, 100 µg (77 µL) of protein extracted from each tumor and normal sample were labeled with Cy5 (Cy5 Mono Reactive Dye; GE Healthcare Biosciences, Uppsala, Sweden), using a scaled-down version of the protocol provided by the manufacturer. The protein extracts were mixed with 20 µL of 0.5 M sodium bicarbonate, pH 8.5. The labeling reaction was carried out for 30 min at room temperature. Labeled samples were purified from free excess dye using the Sigma Spin columns supplied with the antibody microarray kit. The 100 mg labeled protein samples (1 mg/ml) were applied onto the column, and centrifuged first for 2 min and then for 4 min at 800 g. The protein concentration of the recovered samples was adjusted to 1 mg/mL in 100 µL. Before hybridization, the dye-to-protein molar ratio was adjusted to 2.93–4.84 mol of dye/mol of protein according to the manufacturer's instructions. Labeled protein extracts were incubated on Panorama Ab Microarray slides (Sigma-Aldrich) for 30 min with moderate shaking. The slides were washed three times in PBS, in Tween 20 for 5 min, and immersed in water for 2 min. Finally, the slides were air dried and scanned with a laser scanner at the appropriate wave length (Typhoon Trio; GE Healthcare Biosciences).

Data analysis and identification of alterations. Microarray images were analyzed with the Array Vision software version 8.0 (GE Healthcare Biosciences). Fluorescence intensity measurements from each array element were compared with local background, and background subtraction was carried out. The reported value represents the average of all the pixels remaining in the spot, after first removing the pixels with density values that exceeded a threshold value set at four times the absolute deviation value above the median. This measure removes the influence of image artifacts, such as dust particles, on density estimation. The proteins showing ≥2.5-fold difference in expression in more than two cases of three were deemed significant.

Western blotting. Five µg of each protein sample was separated by SDS-PAGE on a 10–12.5% polyacrylamide gradient gel and subsequently blotted on a nitrocellulose membrane. Immunoblot analysis was carried out using the antibodies listed in Table 1 and horseradish peroxidase-conjugated secondary antibodies (1:1000; GE Healthcare Biosciences). Antibody–antigen complexes were visualized with an ECL system (GE Healthcare Biosciences) using LAS 3000 (Fuji Film, Tokyo, Japan). The intensity of the protein bands was quantified and the relative intensity of the examined proteins was calculated on the basis of the intensity of the actin bands on the same membrane.

Immunohistochemistry and tissue microarray. The cellular localization of the proteins was examined immunohistochemically on methanol-fixed, paraffin-embedded tissues using the Dako REAL EnVision Detection System (DAKO, Glostrup, Denmark) following the manufacturer's instructions. In brief, the sections were deparaffinized, dehydrated, and blocked with 0.3% H_2O_2 in methanol for 30 min to remove endogenous peroxidase activity. The sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. The primary antibodies and dilutions used are shown in Table 1.

We obtained two commercial tissue microarrays (ES801; US Biomax, Rockville, MD, USA; and A218(II) AccuMax array; ISU ABXIS, Seoul, Korea) including 40 and 30 esophageal cancer cases with matched normal adjacent tissue, respectively, and used them to examine the expression of the identified proteins and their relationship with the corresponding clinicopathological data. The sex, age, and histological grade were available for all 70 cases on the tissue microarrays, whereas the tumor stage, lymph node metastasis status, distant metastasis status, and tumor size were only available for the 30 cases on the A218(II) array.

mRNA expression analysis. Total RNA was isolated from six ESCC tissues using an Isogen kit (Nippon Gene, Toyama, Japan). The integrity of the RNA was determined on the Agilent 2100 Bioanalyser using the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA). RNA expression analysis was carried out using the GeneChip 3' IVT expression kit (Affymetrix, Santa Clara, CA, USA) according to the supplier's protocol. Labeled targets were hybridized to the GeneChip Human Genome U133 Plus 2.0 Array. Gene expression analysis was carried out using the Affymetrix GeneChip Command Console Software. Experimental data were quantile normalized in Expression Console using the RMA algorithm for probe set summarization. The maximal signal was adopted for subsequent analysis when there were multiple probes corresponding to the same protein.

Statistical analysis. The numerical data were imported into the Expressionist software (Genedata, Basel, Switzerland) for scatter plotting and calculation of the correlation coefficient. The correlation between protein expression and clinicopathological features was evaluated using the Fisher exact test for categorical variables and the Mann–Whitney U-test for continuous variables. P-value differences of <0.05 were considered to be significant. Statistical analyses were carried out using the SPSS 11.0 statistical package (SPSS, Chicago, IL, USA).

Table 1. List of the proteins found to be overexpressed in the microarray analysis and examined by western blotting and immunohistochemistry

| No.	Antibody commercial name	Company	Product no.	Dilution	
				Western blotting	Immunohistochemistry
1	Acetylated protein	Sigma	A5463	1: 100	–
2	Actin	Sigma	A5060	1: 1000	–
3	AP-1	Sigma	A5968	1: 200	1: 5000
4	AP-2a	Sigma	A0844	1: 100	1: 500
5	BAD	BD Biosciences	610391	1: 500	1: 500
6	BUBR1	BD Biosciences	612502	1: 500	1: 500
7	Calbindin-D	Sigma	C7354	1: 250	–
8	Caspase 10	Sigma	C8351	1: 250	1: 500
9	Cdk1	Sigma	C4973	1: 250	1: 500
10	phosph-β-catenin	Sigma	C4231	1: 250	1: 5000
11	Cytokeratin 18	Sigma	C1399	1: 500	–
12	HABH3	Sigma	A8353	1: 250	1: 500
13	hnRNP-U	Sigma	R6278	1: 500	1: 500
14	Mad 2	Sigma	M8694	1: 250	1: 5000
15	NAK	Sigma	N2661	1: 250	1: 50
16	NG2	Sigma	N8912	1: 250	1: 50
17	b-NOS	Sigma	N2280	1: 500	1: 200
18	NGFb	Sigma	N3279	1: 100	–
19	RAB 9	ABR-Affinity Bioreagents	MA3-067	1: 250	1: 200
20	Protein phosphatase 2A alpha	Sigma	P8998	1: 250	–
21	Protein Tyrosine Phosphatase	Sigma	P9109	1: 250	–
22	phospho-Pyk2	Sigma	P7114	1: 250	–
23	SKK2	Abnova	H00005606-M02	1: 100	–
24	Tropomyosin (Sarcimatic)	Sigma	T9283	1: 100	–
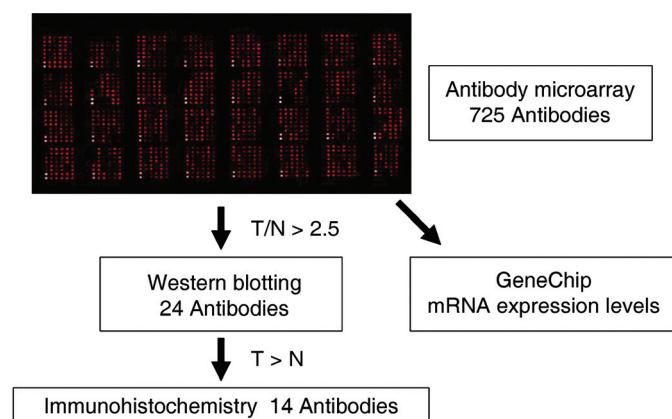
Fig. 1. Scheme showing the experimental flow: the antibody microarray analysis results were validated using western blotting and immunohistochemistry. N, normal; T, tumor.

Results

Antibody microarray performance. The protein contents of surgical specimens from three patients diagnosed with ESCC were extracted. The scanned image of an antibody microarray for a representative tumor extract (1T) is shown in Figure 1. The shape of the spots, the dynamic range of the intensity response, and the signal-to-noise ratio, as well as the positive and negative controls were routinely checked. The reproducibility of the results was monitored by comparing duplicate spots on the same slide. The scatter plot demonstrated that the intensity values of 63.1–94.1% of the antibody spots was scattered within a twofold difference, and that the correlation coefficient was 0.7172–0.9332 in all six samples examined (Fig. 2). Although spot intensity showed high reproducibility for spots with higher intensity in the microarray slide, it showed poor reproducibility

for spots with lower intensity, even when adjacent duplicated spots on the same slide were compared.

Differential protein expression analysis. We initially searched for proteins with upregulated expression in esophageal cancer compared to the corresponding normal reference mucosa. Twenty-four proteins, including two non-unique proteins (whole acetylated proteins and protein tyrosine phosphatases), showed a notable upregulation (more than 2.5-fold change in at least two samples) in the tumor compared to the normal tissues (Table 2). No proteins showed notable downregulation in the tumor tissues compared with their normal reference mucosa. We classified the identified proteins based on their biological function according to Gene Ontology web site and literature searching by the author. Proteins associated with signal transduction were the most frequently identified, followed by those involved in apoptosis, cell cycle, and the cytoskeleton (Table 2). The locus of the identified proteins was variable, and absence of constant chromosomal localization was noted (Table 2).

Western blotting analysis. The 24 proteins identified in the microarray analysis were validated using western blotting. Fourteen of the 24 proteins showed overexpression in the tumor tissues, the results being consistent with the results of microarray analysis. Six proteins showed discordant results; five proteins showed inverse expression compared to the microarray results, whereas actin was found to be consistently expressed in the tumor and normal tissues in western blotting (Table 3; Fig. 3). Four proteins were undetectable in western blotting.

The scattergram (Fig. 4) demonstrates the correlation between the intensity of the duplicate antibody signals of the 24 proteins, in relation to the consistency between western blotting and antibody microarray experiments. The intensity values of the proteins found to be upregulated in tumor tissues in the micro-arrays showed high reproducibility, irrespective of whether these microarray results were discordant ($R = 0.7666$) or concordant ($R = 0.6921$) with western blotting results, or whether these proteins were not detected in western blotting ($R = 0.6317$). We concluded that reproducibility of the antibody microarray
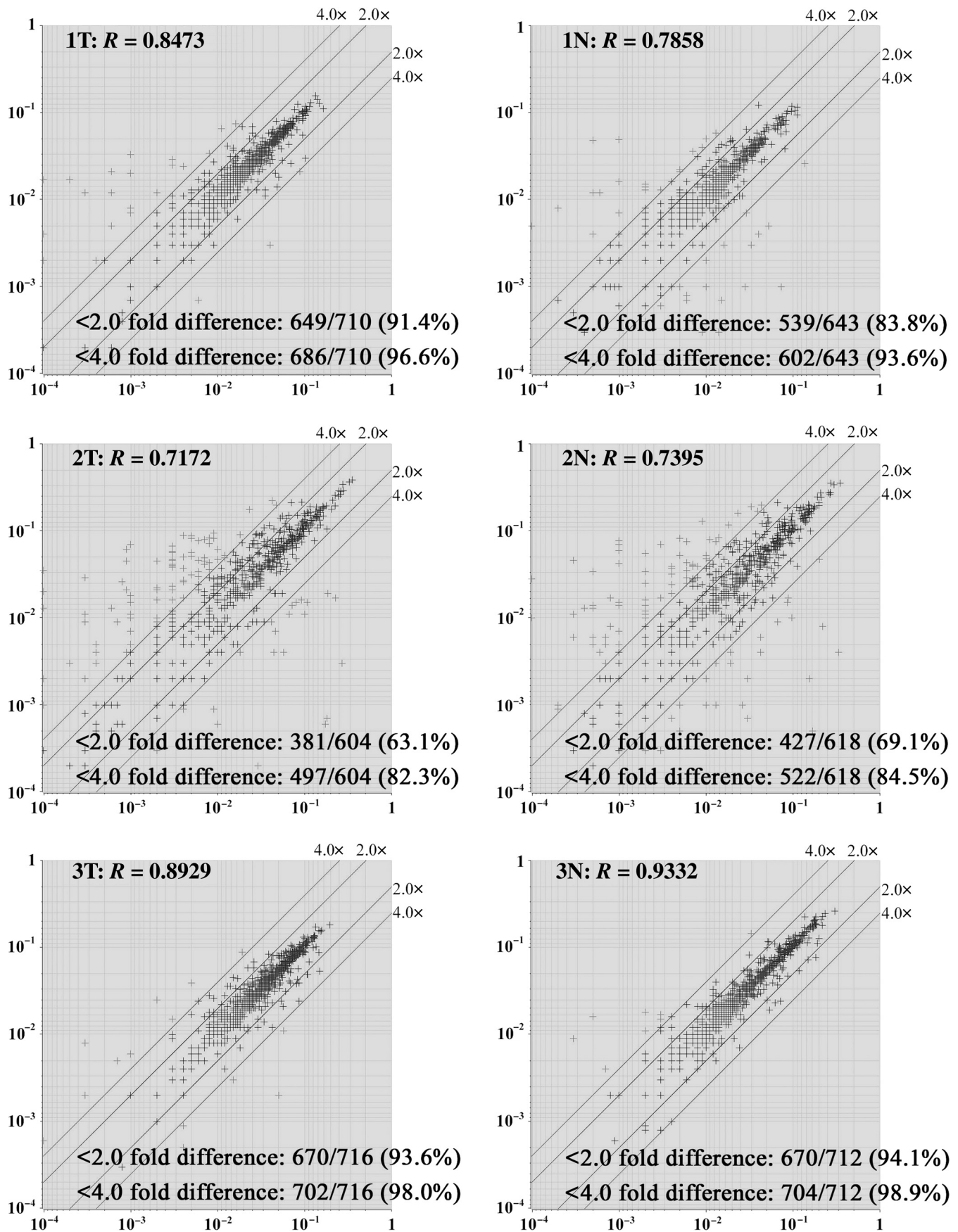
Fig. 2. Reproducibility of the antibody microarray analysis. The scatter plot shows that the intensity values of 63.1–94.1% of the antibodies contained in the microarrays used were scattered within a twofold difference, and that the correlation coefficient (*R*) was 0.7172–0.9332. X-axis, intensity of one spot of duplicate spots; Y-axis, intensity of other spot of duplicate spots.

Table 2. Proteins found to be upregulated in esophageal squamous cell carcinoma tissues in the antibody microarrays

No.	Commercial antibody name	Official protein name	Gene symbol	Entrez gene ID	Biological function	Locus	Cellular localization
1	Acetylated protein	–	–	–	–	–	–
2	Actin	Actin, alpha 1, skeletal muscle	ACTA1	58	Cytoskeleton	1q42.13	Cytoplasm
3	AP-1	jun oncogene	JUN	3725	Cell cycle	1p32-p31	Cytoplasm, nucleus
4	AP-2a	Transcription factor AP-2 alpha	TFAP2A	7020	Transcription	6p24	Nucleus
5	BAD	Bcl-associated death promoter	BAD	572	Apoptosis	11q13.1	Cytoplasm, membrane, mitochondrion
6	BUBR1	BUB 1 budding uninhibited by benzidazoles 1 homolog beta	BUB1B	701	Cell cycle	15q15	Cytoplasm, nucleus
7	Calbindin-D	Calbindin 1	CALB1	793	Calcium-associated	8q21.3–q22.1	Cytoplasm, nucleus
8	Caspase 10	Caspase 10, apoptosis-related cysteine peptidase	CASP10	843	Apoptosis	2q33–q34	Cytoplasm
9	Cdk1	Cell division cycle 2, G1 to S and G2 to M	CDC2	983	Cell cycle	10q21.1	Cytoplasm, nucleus
10	phosph-beta-catenin	Catenin (cadherin associated protein), beta 1	CTNNB1	1499	Signal transduction	3p21	Cytoplasm, nucleus, membrane
11	Cytokeratin 18	Keratin 18	KRT18	3875	Cytoskeleton	12q13	Cytoplasm
12	HABH3	alkB, alkylation repair homolog 3	ALKBH3	221 120	Oxidation reduction	11p11.2	Cytoplasm, nucleus
13	hnRNP-U	Heterogeneous nuclear ribonucleoprotein U	HNRPU	3192	RNA splicing	1q44	Nucleus, cell surface
14	Mad 2	MAD2 mitotic arrest deficient-like 1	MAD2L1	4085	Cell cycle	4q27	Cytoplasm, nucleus
15	NAK	TANK-binding kinase 1	TBK1	29 110	Immune response	12q14.1	Cytoplasm
16	NG2	Chondroitin sulfate proteoglycan 4	CSPG4	1464	Signal transduction	15q24.2	Membrane, cell surface
17	b-NOS	Nitric oxide synthase 1 (neuronal)	NOS1	4842	Oxidation reduction	12q24.2–q24.31	Cytoplasm
18	NGFb	Nerve growth factor	NGFB	4803	Signal transduction	1p13.1	Extracellular region, Golgi
19	RAB 9	RAB9, member RAS oncogene family	RAB9	9367	Signal transduction	Xp22.2	Golgi, lysosome, membrane
20	Protein phosphatase 2A alpha	Protein phosphatase 2, catalytic subunit, alpha isoform	PPP2CA	5515	RNA splicing	5q31.1	Cytoplasm, nucleus, mitochondrion, membrane
21	Protein tyrosine phosphatase	–	–	–	–	–	–
22	phospho-Pyk2	PTK2 protein tyrosine kinase 2 beta	PTK2B	2185	Apoptosis	8p21.1	Cytoplasm, membrane
23	SKK2	Mitogen-activated protein kinase kinase 3	MAP2K3	5606	Signal transduction	17q11.2	Protein complex
24	Tropomyosin (Sarcimatic)	Tropomyosin 1 (alpha)	TPM1	7168	Cytoskeleton	15q22.1	Cytoplasm, nucleus

experiments may not guarantee concordant results between western blotting and antibody microarray experiments.

Correlation between microarray and western blotting data. The quantitative correlation between the microarray and western blotting data was examined. Each tumor tissue's data were normalized by the normal counterpart's data. The correlation coefficient for AP-1 and AP-2α could not be calculated due to missing microarray values. The 14 proteins overexpressed in tumor tissues in western blotting included proteins with a high correlation coefficient, such as Mad2 ($R = 0.934$) and NAK ($R = 0.763$), as well as proteins with a poor correlative relationship, such as hABH3 ($R = -0.981$) and NG2 ($R = -0.933$) (Table 3; Fig. 5).

Immunohistochemistry. We carried out immunohistochemistry to verify the abundance and cellular localization of the 14 proteins with high expression levels in ESCC as revealed by western blotting (Table 3). The expression of six proteins (AP-1, BUBR1, caspase 10, Cdk1, NAK, and Mad2), either cytoplasmic or nuclear, was stronger in ESCC compared with normal epithelium. Nuclear expression of heterogeneous nuclear ribonucleoprotein U (hn-RNP-U) was observed in all tumor and normal tissues. The expression of Bcl-associated death promoter (BAD) and AP-2α was higher in infiltrating inflammatory cells compared with the tumor stroma (Fig. 6). The expression of p-β-catenin, HABH3, and member RAS oncogene family (RAB) was similar in tumor and normal tissues (data not shown). Nitric oxide synthase 1 (b-NOS) and NG2 expression were not detected immunohistochemically (data not shown).

Of the six proteins with stronger immunohistochemical expression in ESCC, tissue microarray analysis showed that BUBR1, Mad2, AP-1, caspase 10, NAK, and Cdk1 were over-expressed in 80, 86, 73, 73, 63, and 90% of ESCC patients, respectively, compared with normal tissues. There was no association between BUBR1, AP-1, caspase 10, or Cdk1 expression with clinicopathological characteristics. Overexpression of Mad2 and NAK was infrequent in tumors histologically categorized as grade 3 or with lymph node metastases, respectively (Table 4).

mRNA expression. The mRNA expression levels of 22 proteins identified by antibody microarray as overexpressed in tumors

Table 3. Summary of the results of western blotting and immunohistochemistry

No.	Antibody commercial name	WB	R (WB array)	Ihc localization
1	Acetylated protein	Undetectable	ND	ND
2	Actin	T = N	ND	ND
3	AP-1	T > N	†	Cytoplasm, nucleus
4	AP-2a	T > N	†	Inflamatory cells
5	BAD	T > N	0.351	Inflamatory cells
6	BUBR1	T > N	−0.178	Cytoplasm
7	Calbindin-D	T < N	ND	ND
8	Caspase 10	T > N	−0.572	Cytoplasm
9	Cdk1	T > N	0.583	Nucleus
10	phosph-beta-catenin	T > N	−0.699	Cytoplasm
11	Cytokeratin 18	T < N	ND	ND
12	HABH3	T > N	−0.981	Nucleus
13	hnRNP-U	T > N	−0.458	Nucleus
14	Mad 2	T > N	0.934	Cytoplasm
15	NAK	T > N	0.763	Cytoplasm
16	NG2	T > N	−0.933	Undetectable
17	b-NOS	T > N	0.708	Undetectable
18	NGFb	Undetectable	ND	ND
19	RAB 9	T > N	0.805	Cytoplasm
20	Protein phosphatase 2A alpha	T < N	ND	ND
21	Protein tyrosine phosphatase	T < N	ND	ND
22	phospho-Pyk2	Undetectable	ND	ND
23	SKK2	Undetectable	ND	ND
24	Tropomyosin (Sarcimatic)	T < N	ND	ND

†Not calculated due to missing array data. IHC, immunohistochemistry; ND, not done; *R*, correlation coefficiency of the western blotting compared to the microarray results; WB, western blotting.
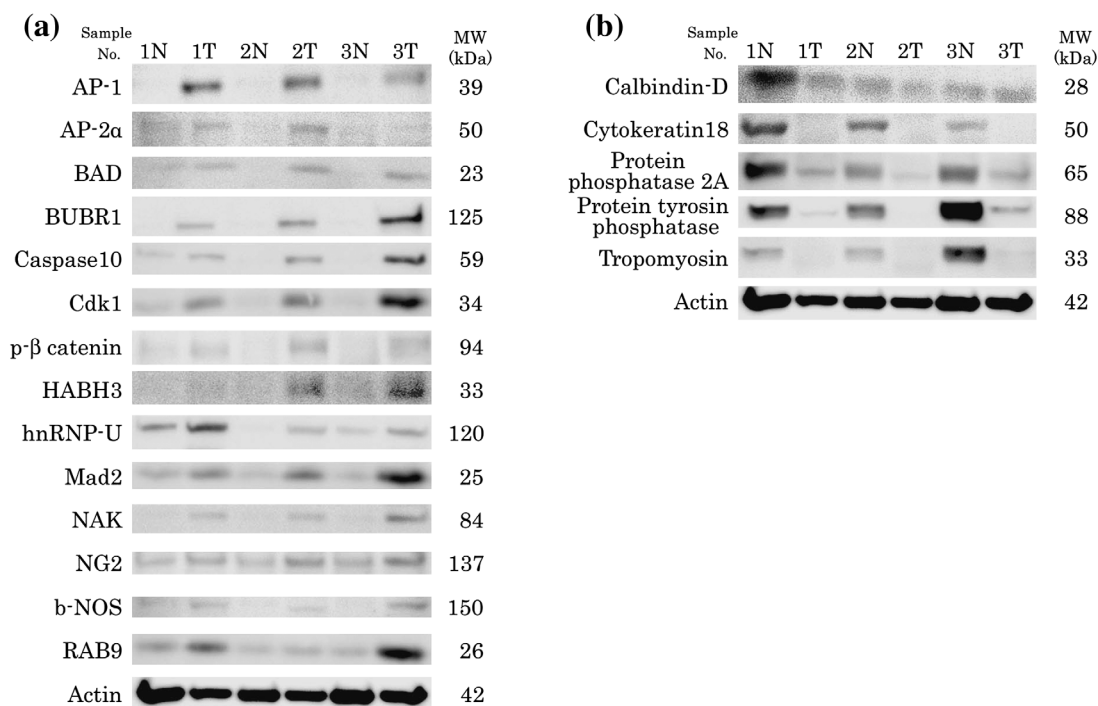

Fig. 3. Western blotting results. (a) Fourteen of the 24 proteins identified in the microarray analysis as being overexpressed in tumor tissues showed concordant results in western blotting analysis. (b) Five proteins showed discordant results.

were examined and compared with western blotting results (Fig. 7). Among the 14 proteins found to be overexpressed in ESCC in western blotting analysis, nine (64%) proteins were also overexpressed at the mRNA level. The expression of two (50%) of four proteins found to be lower in ESCC by western blotting was also lower at the mRNA level. Discordance between the mRNA and western blotting results was observed in approximately 40% of the proteins studied.
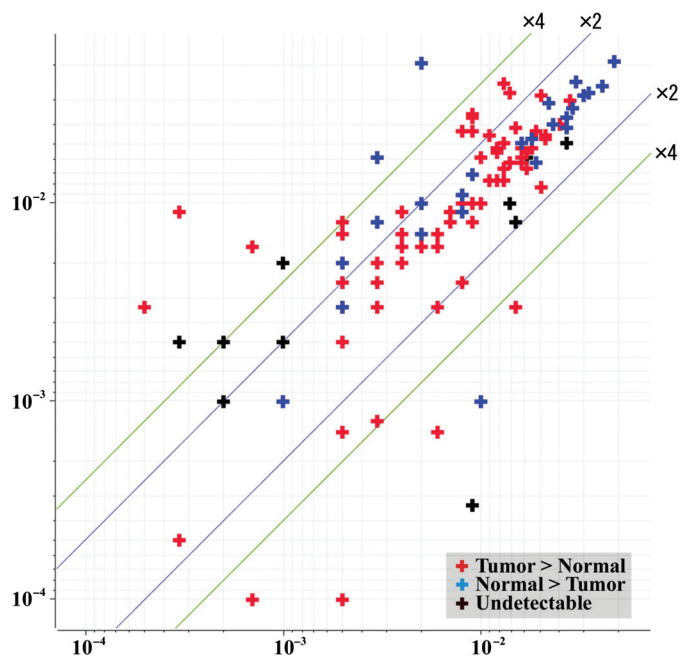
Fig. 4. The scattergram demonstrates the correlation between the intensity of duplicate antibody signals of the 24 proteins found to be overexpressed in the microarray analysis. The intensity values of the proteins found to be overexpressed in tumor tissues in the microarrays showed high reproducibility, irrespective of whether these microarray results were discordant ($R = 0.7666$) or concordant ($R = 0.6921$) with western blotting results, or whether these proteins were not detected by western blotting ($R = 0.6317$). X-axis, intensity of one spot of duplicate spots; Y-axis, intensity of other spot of duplicate spots.

Discussion

The study of cancer through the use of antibody microarrays provides valuable information on protein expression levels, isoforms expressed, and post-translational modifications concerning the particular cancer studied. Furthermore, as many reports have demonstrated discordance between mRNA and protein expression,[15–17] which concerned approximately 40% of proteins in this study, the information provided more closely reflects cancer phenotypes. As well as in separation-based proteomics such as that using two-dimensional gel electrophoresis,[7,18,19] antibody microarrays have been used in the study of colorectal,[20] breast,[21,22] pancreatic,[23,24] bladder,[25] and prostate cancer,[26] squamous cell carcinoma of the oral cavity,[27,28] hepatocellular carcinoma,[29] and gastric cancer.[30] Although several proteomic studies have been reported,[7,8] antibody microarrays have not been used to date in esophageal cancer.

Microarray analysis identified 24 proteins that were overexpressed in the tumor tissues compared with the corresponding normal tissues. Examination of these proteins by western blotting and immunohistochemistry validated these results for six of these proteins. Of the six proteins, Mad2 and BubR1 are crucial components of a functional mitotic checkpoint by organizing spindle assembly.[31] Overexpression of the *Mad2* gene has been reported in nasopharyngeal,[32] gastric,[33] ovarian,[34] colorectal,[35] hepatocellular,[36] and esophageal cancer,[37] whereas BubR1 is overexpressed in colorectal,[38] gastric,[39] and esophageal cancer.[37] Tanaka *et al.* reported that Mad2 and BubR1 are coordinately overexpressed in ESCC, and were overexpressed in 89 and 95% of ESCC, respectively, compared with normal tissues.[37] Although they reported no correlation between any clinicopathological

Table 4. Tissue microarray results

Variable	n	BUBR1	Mad2	AP-1	Caspase 10	NAK	Cdk1
All cases	70	56 (80%)	60 (86%)	51 (73%)	51 (73%)	44 (63%)	63 (90%)
Sex							
Male	55	44 (80%)	49 (89%)	40 (73%)	40 (73%)	33 (60%)	49 (89%)
Female	15	12 (80%)	11 (73%)	11 (73%)	11 (73%)	11 (73%)	15 (100%)
Age (years)							
<60	38	29 (76%)	32 (84%)	27 (71%)	25 (66%)	20 (53%)	34 (89%)
≥60	32	27 (84%)	28 (88%)	24 (75%)	26 (81%)	24 (75%)	29 (91%)
Histological grade							
1	16	12 (75%)	16 (100%)*	13 (81%)	9 (56%)	6 (38%)	14 (88%)
2	42	33 (79%)	39 (93%)*	31 (74%)	33 (79%)	29 (69%)	39 (93%)
3	9	8 (89%)	2 (22%)*	4 (44%)	6 (67%)	7 (78%)	8 (89%)
X	3	3 (100%)	3 (100%)	3 (100%)	3 (100%)	2 (67%)	2 (67%)
Tumor stage							
T1	12	10 (83%)	12 (100%)	9 (75%)	9 (75%)	9 (75%)	11 (92%)
T2	7	5 (71%)	6 (86%)	7 (100%)	6 (86%)	4 (57%)	7 (100%)
T3	10	8 (80%)	8 (80%)	8 (80%)	10 (100%)	5 (50%)	9 (90%)
T4	1	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)	1 (100%)
Lymph node metastasis							
Negative	17	13 (76%)	15 (88%)	12 (71%)	13 (76%)	14 (82%)*	16 (94%)
Positive	13	10 (77%)	12 (92%)	13 (100%)	12 (92%)	4 (31%)*	12 (92%)
Distant metastasis							
Negative	25	19 (76%)	23 (92%)	21 (84%)	20 (80%)	15 (60%)	23 (92%)
Positive	5	4 (80%)	4 (80%)	4 (80%)	5 (100%)	3 (60%)	5 (100%)
Tumor size (mm)							
<40	14	9 (64%)	12 (86%)	11 (79%)	10 (71%)	10 (71%)	14 (100%)
≥40	16	14 (88%)	15 (94%)	14 (88%)	15 (94%)	8 (50%)	14 (88%)

*Statistically significant differences ($P < 0.05$). BUBR, budding uninhibited by benzidazoles 1 homolog beta; Mad, mitotic arrest deficient-like 1; AP, activator protein; NAK, TANK-binding kinase 1; Cdk, cyclin-dependent kinase.
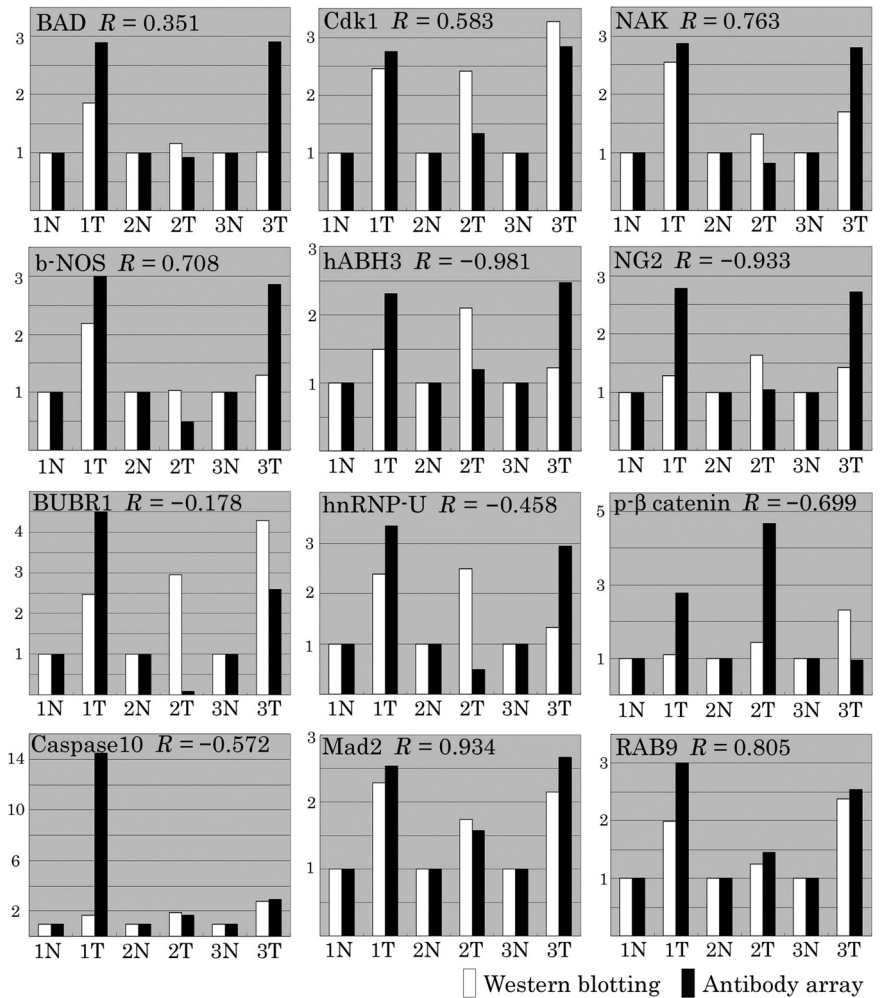
Fig. 5. Comparison of the microarray with the western blotting data. The correlation coefficient of the 14 proteins found to be overexpressed in tumor tissues in western blotting was calculated. Normalization of each tumor tissue's data was carried out using the normal counterpart's data.

☐ Western blotting ■ Antibody array

data, including the histological grade and the expression of these proteins, we identified a correlation between Mad2 expression and histological grade (Table 4). This correlation has also been reported in gastric cancer[33] and hence is a finding that warrants further investigation. In this study, we detected overexpression of Mad2 and BubR1, which were included in cell cycle regulation, in ESCC in different cohorts. Recently, the relationship between the mitotic checkpoint and drug sensitivity has been the subject of extensive investigations.[40] These proteins may be considered as important determinants of the efficacy of microtubule-targeting drugs in killing cancer cells.

Cdk1 is a member of the CDK family, which regulates phase transitions and checkpoints within the cell cycle.[41,42] Under non-neoplastic conditions, Cdk1 is localized to the nucleus where it regulates cell cycle progression as a subunit of the M-phase promoting factor, together with a cyclin B subunit. Within the normal esophageal mucosa, Cdk1 is localized to the parabasal cell layer, where the majority of normal cellular proliferation occurs.[43] In this study, the fact that these three proteins serving crucial roles in M-phase regulation were concurrently identified may indicate that one or all of them may contribute significantly to the carcinogenesis of ESCC.

The remaining three proteins, AP-1, caspase 10, and NAK, are associated with NF-κB, which is a signal transduction factor that has emerged as an important modulator of altered gene programs and malignant phenotype in the development of cancer.[44] In one esophageal adenocarcinoma study, 61% of resected tumors displayed NF-κB immunoreactivity and 87.5% of the

NF-κB-positive tumors were Stage IIb or III.[45] Constitutive activation of the NF-κB and AP-1 signal transduction pathways have been identified as prominent events promoting tumor progression of hemopoietic and solid malignancies.[46–48] Overexpression of a caspase 10 subclass dramatically enhances NF-κB activity in a dose- and time-dependent manner.[49] NAK phosphorylates a NF-κB subunit and increases its activity.[50] In this study, the correlation between underexpression of NAK and the presence of lymph node metastases was revealed (Table 4); although NAK has been reported as a mediator of tumor angiogenesis,[51] this correlation had not been reported to date and, again, additional investigation is necessary. To the best of our knowledge, aberrant expression of these proteins has not been previously reported in ESCC. Our results also suggest that these members of the NF-κB signaling pathway may play an important role in the carcinogenesis of ESCC.

In this study, the reproducibility of the antibody microarray results was poor (Fig. 2) and varied between the experiments, with the correlation coefficient ranging from 0.7172 to 0.9332. It remains a challenge to improve the reproducibility of the results obtained using antibody microarrays such as the ones in our study. To achieve this, Ellmark *et al*. used eight replicates in a microarray and excluded the two highest and two lowest replicates, and each data point represented the mean value of the remaining four replicates.[30] When space allows, the antibody microarrays should therefore be spotted with more replicates in the same microarray to improve reproducibility. Furthermore, as the degree of reproducibility was different based on the signal
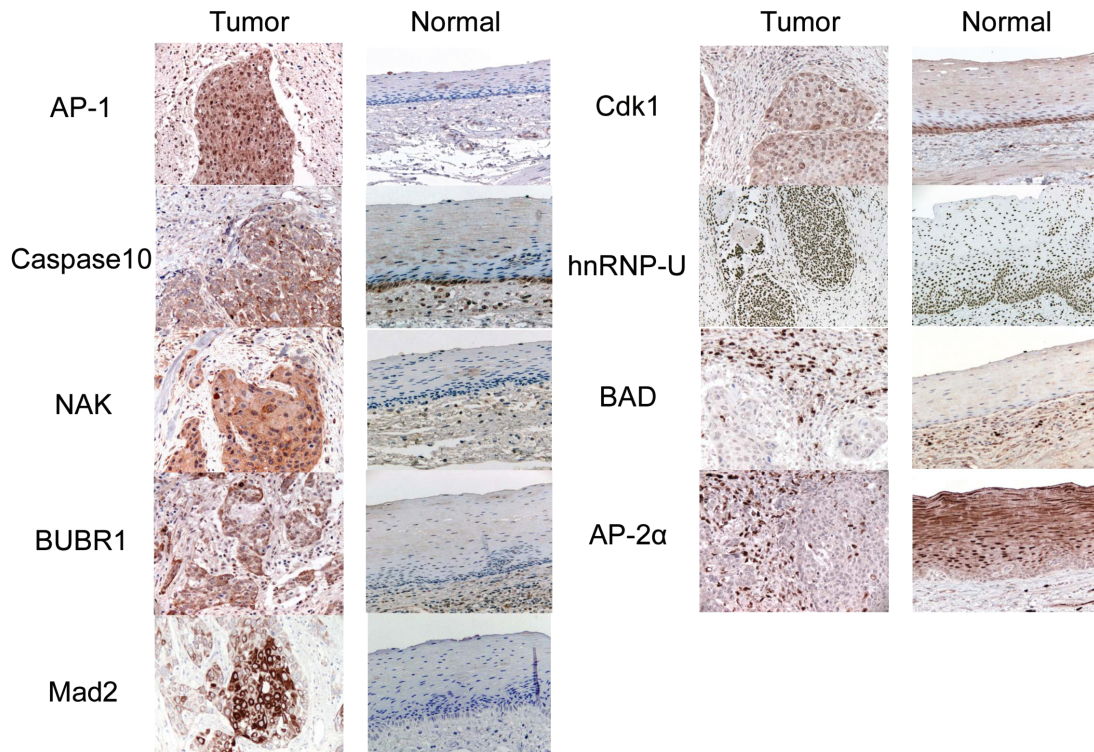
Fig. 6. Immunohistochemistry. The expression of six proteins (activator protein [AP]-1, BUBR1, caspase 10, cyclin-dependent kinase [Cdk] 1, nuclear factor-κB-activating kinase [NAK], and Mad2), either cytoplasmic or nuclear, was stronger in esophageal squamous cell carcinoma compared with normal epithelium. Nuclear expression of hn-RNP-U was observed in all tumor and normal tissues. The expression of BAD and AP-2α was higher in infiltrating inflammatory cells compared with the tumor stroma. BUBR, budding uninhibited by benzidazoles 1 homolog beta; Mad, mitotic arrest deficient-like 1; hn-RNP-U, heterogeneous nuclear ribonucleoprotein U; BAD, Bcl-associated death promoter.
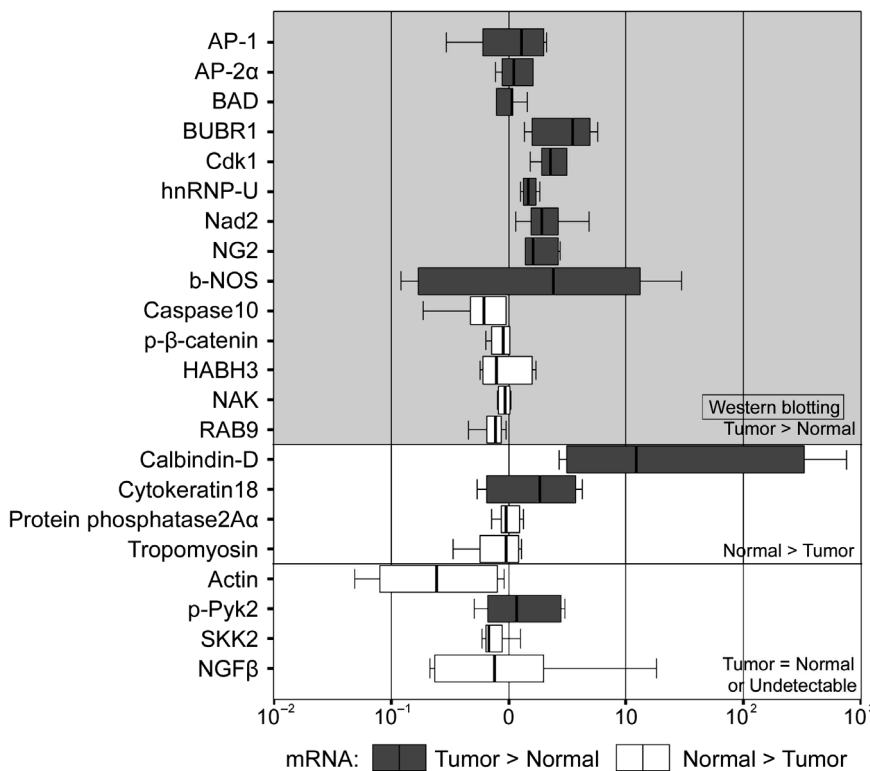

Fig. 7. The mRNA expression levels of the 22 identified proteins. Among the 14 proteins overexpressed in esophageal squamous cell carcinoma (ESCC) by western blotting analysis, nine were also found to be overexpressed at the mRNA level. The expression of two of four proteins found to be lower in ESCC in western blotting was also lower at the mRNA level.

intensity, the threshold may also need to be changed as a function of signal intensity.

As spots with lower signal intensity had poor reproducibility (Fig. 2), it may be better to pre-exclude them from data analysis. The amount and affinity of the antibodies in the microarray should be best optimized so that the dynamic range of all spots is within a linearly measurable range. However, the affinity to antigens is different between antibodies. In addition, the amount of individual antigens is also variable between the samples. It seems that these problems may be inherent to antibody microarray experiments. Therefore, extensive validation studies by other methods should be carried out for results obtained by antibody microarrays.

As tumor tissues contain many types of tumor and non-tumor cells, recovery of specific cell populations by laser microdissection prior to protein extraction is generally required for accurate expression analysis. However, as 100 μg of each protein is required for every antibody microarray experiment, and laser microdissection can collect a small amount of protein, we cannot avoid using homogenized tissue samples. The advantages and limitations of antibody microarrays, western blotting, and immunohistochemistry in the analysis of protein expression are discussed in the Supplemental text. In our study, two proteins identified as being overexpressed in tumor tissues were shown to derive from infiltrating inflammatory cells in the tumor stroma rather than the tumor itself, a finding that stresses the need for immunohistochemical studies in order to localize the identified proteins in the tissues.

In conclusion, through the use of antibody microarrays we identified a novel association of mitotic checkpoint gene products and the NF-κB pathway with esophageal cancer. These proteins will not only be useful in better understanding the molecular background of ESCC but will also be candidates for biomarkers or therapeutic targets. Although antibody microarrays generate expression data in a high-throughput way, validation of the results by other methods is required due to its limited reproducibility.

Acknowledgments

Disclosure Statement

The authors declare that they have no competing interests.

Abbreviations

AP	activator protein
BubR	budding uninhibited by benzidazoles 1 homolog beta
Cdk	cyclin-dependent kinase
ESCC	esophageal squamous cell carcinoma
HABH	alkylation repair homolog
Mad	mitotic arrest deficient-like 1
NAK	nuclear factor-κB-activating kinase
NF	nuclear factor
NG	neurite growth

References

1 Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; **80**: 827–41.

2 Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; **83**: 18–29.

3 Ilson DH. Oesophageal cancer: new developments in systemic therapy. *Cancer Treat Rev* 2003; **29**: 525–32.

4 Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241–52.

5 Mariette C, Balon JM, Piessen G, Fabre S, Van Seuningen I, Triboulet JP. Pattern of recurrence following complete resection of esophageal carcinoma and factors predictive of recurrent disease. *Cancer* 2003; **97**: 1616–23.

6 Metzger R, Schneider PM, Warnecke-Eberz U, Brabender J, Holscher AH. Molecular biology of esophageal cancer. *Onkologie* 2004; **27**: 200–6.

7 Hatakeyama H, Kondo T, Fujii K *et al*. Protein clusters associated with carcinogenesis, histological differentiation and nodal metastasis in esophageal cancer. *Proteomics* 2006; **6**: 6300–16.

8 Liu WL, Zhang G, Wang JY *et al*. Proteomics-based identification of autoantibody against CDC25B as a novel serum marker in esophageal squamous cell carcinoma. *Biochem Biophys Res Commun* 2008; **375**: 440–5.

9 Homs MY, Kuipers EJ, Siersema PD. Palliative therapy. *J Surg Oncol* 2005; **92**: 246–56.

10 Katlic MR, Wilkins EW Jr, Grillo HC. Three decades of treatment of esophageal squamous carcinoma at the Massachusetts General Hospital. *J Thorac Cardiovasc Surg* 1990; **99**: 929–38.

11 Brockmann JG, St Nottberg H, Glodny B, Heinecke A, Senninger NJ. CYFRA 21-1 serum analysis in patients with esophageal cancer. *Clin Cancer Res* 2000; **6**: 4249–52.

12 Crnogorac-Jurcevic T, Gangeswaran R, Bhakta V *et al*. Proteomic analysis of chronic pancreatitis and pancreatic adenocarcinoma. *Gastroenterology* 2005; **129**: 1454–63.

13 Ponten F, Jirstrom K, Uhlen M. The Human Protein Atlas – a tool for pathology. *J Pathol* 2008; **216**: 387–93.

14 Sobin L, Wittekind C. *UICC TNM Classification of Malignant Tumors*, 6th edn. New York: Wiley-Liss, 2002.

15 Chen G, Gharib TG, Huang CC *et al*. Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* 2002; **1**: 304–13.

16 Varambally S, Yu J, Laxman B *et al*. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell* 2005; **8**: 393–406.

17 Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 1999; **19**: 1720–30.

18 Uemura N, Nakanishi Y, Kato H *et al*. Transglutaminase 3 as a prognostic biomarker in esophageal cancer revealed by proteomics. *Int J Cancer* 2009; **124**: 2106–15.

19 Nishimori T, Tomonaga T, Matsushita K *et al*. Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin. *Proteomics* 2006; **6**: 1011–18.

20 Madoz-Gurpide J, Canamero M, Sanchez L, Solano J, Alfonso P, Casal JI. A proteomics analysis of cell signaling alterations in colorectal cancer. *Mol Cell Proteomics* 2007; **6**: 2150–64.

21 Smith L, Watson MB, O'Kane SL, Drew PJ, Lind MJ, Cawkwell L. The analysis of doxorubicin resistance in human breast cancer cells using antibody microarrays. *Mol Cancer Ther* 2006; **5**: 2115–20.

22 Celis JE, Moreira JM, Cabezon T *et al*. Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial–adipocyte stromal cell interactions. *Mol Cell Proteomics* 2005; **4**: 492–522.

23 Ingvarsson J, Wingren C, Carlsson A *et al*. Detection of pancreatic cancer using antibody microarray-based serum protein profiling. *Proteomics* 2008; **8**: 2211–19.

24 Orchekowski R, Hamelinck D, Li L *et al*. Antibody microarray profiling reveals individual and combined serum proteins associated with pancreatic cancer. *Cancer Res* 2005; **65**: 11 193–202.

25 Sanchez-Carbayo M, Socci ND, Lozano JJ, Haab BB, Cordon-Cardo C. Profiling bladder cancer using targeted antibody arrays. *Am J Pathol* 2006; **168**: 93–103.

26 Miller JC, Zhou H, Kwekel J *et al*. Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* 2003; **3**: 56–63.

27 Knezevic V, Leethanakul C, Bichsel VE *et al*. Proteomic profiling of the cancer microenvironment by antibody arrays. *Proteomics* 2001; **1**: 1271–8.

28 Weber A, Hengge UR, Stricker I *et al*. Protein microarrays for the detection of biomarkers in head and neck squamous cell carcinomas. *Hum Pathol* 2007; **38**: 228–38.

29 Tannapfel A, Anhalt K, Hausermann P *et al*. Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. *J Pathol* 2003; **201**: 238–49.

30 Ellmark P, Ingvarsson J, Carlsson A, Lundin BS, Wingren C, Borrebaeck CA. Identification of protein expression signatures associated with *Helicobacter pylori* infection and gastric adenocarcinoma using recombinant antibody microarrays. *Mol Cell Proteomics* 2006; **5**: 1638–46.

31 Hoyt MA. A new view of the spindle checkpoint. *J Cell Biol* 2001; **154**: 909–11.

32 Wang X, Jin DY, Wong YC *et al*. Correlation of defective mitotic checkpoint with aberrantly reduced expression of MAD2 protein in nasopharyngeal carcinoma cells. *Carcinogenesis* 2000; **21**: 2293–7.

33 Tanaka K, Nishioka J, Kato K *et al*. Mitotic checkpoint protein hsMAD2 as a marker predicting liver metastasis of human gastric cancers. *Jpn J Cancer Res* 2001; **92**: 952–8.

34 Wang X, Jin DY, Ng RW *et al*. Significance of MAD2 expression to mitotic checkpoint control in ovarian cancer cells. *Cancer Res* 2002; **62**: 1662–8.

35 Li GQ, Li H, Zhang HF. Mad2 and p53 expression profiles in colorectal cancer and its clinical significance. *World J Gastroenterol* 2003; **9**: 1972–5.

36 Jeong SJ, Shin HJ, Kim SJ *et al*. Transcriptional abnormality of the hsMAD2 mitotic checkpoint gene is a potential link to hepatocellular carcinogenesis. *Cancer Res* 2004; **64**: 8666–73.

37 Tanaka K, Mohri Y, Ohi M *et al*. Mitotic checkpoint genes, hsMAD2 and BubR1, in oesophageal squamous cancer cells and their association with 5-fluorouracil and cisplatin-based radiochemotherapy. *Clin Oncol (R Coll Radiol)* 2008; **20**: 639–46.

38 Shichiri M, Yoshinaga K, Hisatomi H, Sugihara K, Hirata Y. Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival. *Cancer Res* 2002; **62**: 13–17.

39 Grabsch H, Takeno S, Parsons WJ *et al*. Overexpression of the mitotic checkpoint genes BUB1, BUBR1, and BUB3 in gastric cancer – association with tumour cell proliferation. *J Pathol* 2003; **200**: 16–22.

40 Lee EA, Keutmann MK, Dowling ML, Harris E, Chan G, Kao GD. Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells. *Mol Cancer Ther* 2004; **3**: 661–9.

41 Doree M, Hunt T. From Cdc2 to Cdk1: when did the cell cycle kinase join its cyclin partner? *J Cell Sci* 2002; **115**: 2461–4.

42 Murray AW. Recycling the cell cycle: cyclins revisited. *Cell* 2004; **116**: 221–34.

43 Hansel DE, Dhara S, Huang RC *et al*. CDC2/CDK1 expression in esophageal adenocarcinoma and precursor lesions serves as a diagnostic and cancer progression marker and potential novel drug target. *Am J Surg Pathol* 2005; **29**: 390–9.

44 Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. *Clin Cancer Res* 2007; **13**: 1076–82.

45 Abdel-Latif MM, O'Riordan J, Windle HJ *et al*. NF-kappaB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann Surg* 2004; **239**: 491–500.

46 Aggarwal BB. Nuclear factor-kappaB: the enemy within. *Cancer Cell* 2004; **6**: 203–8.

47 Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002; **4**: E131–6.

48 Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 2003; **3**: 859–68.

49 Wang H, Wang P, Sun X *et al*. Cloning and characterization of a novel caspase-10 isoform that activates NF-kappa B activity. *Biochim Biophys Acta* 2007; **1770**: 1528–37.

50 Bouwmeester T, Bauch A, Ruffner H *et al*. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat Cell Biol* 2004; **6**: 97–105.

51 Czabanka M, Korherr C, Brinkmann U, Vajkoczy P. Influence of TBK-1 on tumor angiogenesis and microvascular inflammation. *Front Biosci* 2008; **13**: 7243–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplemental text

We compared the protein expression level of the samples examined using three widely used methods that use antibodies, namely antibody microarrays, western blotting, and immunohistochemistry, as each methodology has advantages and limitations. Antibody microarrays can generate expression data of hundreds or thousands of proteins in a single experiment. However, antibody microarrays measure the total amount of proteins examined, and the expression of isoforms and variants cannot be evaluated. Western blotting separates proteins first according to their molecular weight and the separated proteins then react with antibodies. The advantage of western blotting is that it measures the expression level of protein isoforms and variants even using antibodies that react with all protein forms. Improving throughputness is the challenge of western blotting for proteomic studies. The unique advantage of immunohistochemistry is that it provides detailed data on the localization and distribution of the proteins examined in the cells and tissues. The acquisition quantitative data relies on objective observation in immunohistochemistry. The discordant results observed in this study between these methodologies may be attributable to the reactivity of the antibodies used. The physical and chemical status of proteins may vary in these methods and the antibodies may react with them in varying ways. The use of multiple antibodies against single antigens may be helpful in measuring the expression level of proteins.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.