Differential Protein Expression of Lymph Node Metastases of Papillary Thyroid Carcinoma Harboring the *BRAF* Mutation

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Abstract. *Background: The prognostic role of the T1799A BRAF mutation is controversial. We investigated the protein expression in papillary thyroid carcinoma (PTCs) samples harboring the specific mutation using proteomic tools. Materials and Methods: We performed two-dimensional gel electrophoresis to identify differential protein expression regarding lymph node metastasis (LNM). Proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Immunohistochemical staining was performed for 38 PTCs harboring the mutation. We validated the association between these proteins and clinicopathological factors in a test set of 121 PTCs. Results: The expression of vimentin was increased in PTCs with LNM, but the one for HSP60, SOD2 and PEBP1 was increased in samples without LNM. HSP60 protein was up-regulated in PTCs without LNM (84.2% vs. 36.8%. p=0.003) and in PTCs without LNM harboring the mutation (58% vs. 41.8%. p=0.003) in the test set as shown by immunohistochemical staining. Conclusion: HSP60 protein expression may inhibit LNM in PTCs harboring the BRAF mutation and may be a useful prognostic marker.*

The incidence of thyroid cancer has increased worldwide, and thyroid cancer is the most common cancer among females in South Korea (1, 2). The most common subtype of thyroid cancer is papillary thyroid carcinoma (PTC). Although most patients with PTC have good prognosis, some PTC cases exhibit aggressive clinical behavior. Thus, current prognostic systems often fail to reliably predict the disease course.

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The precise molecular mechanisms promoting the progression of PTC have not been well-elucidated. The T1799A *BRAF*^V600E mutation (*BRAF* mutation) is the most common genetic alteration in PTC and tends to be associated with clinicopathologically high-risk factors, recurrence, and loss of iodine avidity (3, 4). There are controversies as to whether theses tendencies are similar across ethnic groups, especially in Koreans, for whom *BRAF* mutations are more prevalent (5-7). Recent studies suggest that aberrant methylation of specific genes and subsequent silencing of gene expression may play an important role in the progression and aggressiveness of PTC (8-11). Effects of the *BRAF* mutation can be variable due to changes in the downstream steps in the RET/PTC, Ras, Raf, MAP kinase/ERK pathway (MAPK pathway) (12). A few auxiliary prognostic markers to *BRAF* mutation, such as mitogen-inducible factor-6 and insulin-like growth factor binding protein-7, have been identified, and most of these markers are found to have significance at the genetic level (13-15).

The use of proteomics has received attention because neither genetic nor mRNA analysis can elucidate the actual presence of particular proteins or their quantity. Thus, to find practical prognostic factors, it may be important to search at the protein level.

This study aimed to identify auxiliary prognostic protein markers from PTC samples harboring *BRAF* mutations that grouped PTC cases by lymph node metastasis (LNM) status. Proteomic tools, such as a two-dimensional gel electrophoresis (2D-GE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), were used to conduct the study. In addition, to validate the identified marker and clarify its clinical significance, we evaluated it in a test set of PTC samples.

Materials and Methods

Study cohort and specimen procurement. Six snap-frozen PTC tissues were retrieved from the operating theatre, and *BRAF* mutations were identified within those samples. After identification of candidate proteins, 38 paraffin-embedded PTC tissue samples

were selected for immunohistochemical validation of protein expression. The samples in the validation set all carried the *BRAF* mutation. Nineteen validation samples were from PTC cases with LNM, while the other 19 were from cases without LNM. After the validation experiments, a test set of 121 random PTC samples was constructed. All specimens were obtained after informed consent and the study was approved by the ethics committee of our institution.

Detection of the BRAF^V600E mutation. Polymerase chain reaction (PCR) was performed using 5 μl of genomic DNA extract, 1 U Taq polymerase, 0.25 mM of each dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl$_2$ and 10 pmol primers in a final volume of 20 μl. Cycling was carried out as follows: initial denaturation at 95°C for 5 min, followed by 34 cycles of: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec. A final extension was performed at 72°C for 10 min. The forward PCR primer was 5'GCT TGC TCT GAT AGG AAA ATG AG3' and the reverse primer was 5'GTA ACT CAG CAG CAT CTC AGG3'. After purification of the PCR products, direct DNA sequencing was performed using the MegaBACE 100 sequencing analysis system (Amersham, Piscataway, NJ, USA) using a standard protocol. As positive controls for the *BRAF* mutation, we used the human anaplastic thyroid cancer cell line ARO (ATCC, Manassas, VA, USA), which is known to be heterozygous for the *BRAF* mutation. As a negative control, we used the MCF-7 (ATCC) human breast cancer cell line, which bears no BRAF mutation.

Proteomic analysis. Two-dimensional gel electrophoresis 2D-GE. We performed 2D-GE with extracted protein in urea-lysis buffer to compare protein expression between PTC samples from cases with LNM (n=2) and PTC samples from cases without LNM (n=2). The proteins were extracted from snap-frozen PTC tissues harboring the *BRAF* mutation.

Thyroid tissue samples were homogenized in 400 μl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris-HCl, 65 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor cocktail, 0.5% carrier ampholyte and incubated for 45 min at room temperature. After centrifugation at 12,000 g for 15 min at 12°C, 150 μg of proteins were dissolved with a rehydration buffer (8 M urea, 4% CHAPS, 10 mM DTT, 0.5% carrier ampholyte). Using 250 μl of each paired preparation, 13 cm immobilized pH 3-10 non-linear gradient strips were rehydrated. Once isoelectric focusing (first dimension) was completed, the strips were equilibrated in an equilibration buffer (25 mM Tris-HCl, 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 130 mM DTT) for 10 min. The second dimension was performed by using 10% polyacrylamide gels at 100 V constant current per gel. The gels were stained using a colloidal blue staining kit (Invitrogen, Carlsbad, CA, USA) for 24 h and destained with deionized water.

All protein spots that showed differences in intensity were investigated and defined as putative candidates for LNM-associated proteins (LMAPs). These were confirmed by cross-comparison of all LMAPs in 2D-GE gels. Gel comparisons were performed using the TOPSPOT image analysis software program (http://www.mpiib-berlin.mpg.de/2D-PAGE/). Spot intensities were normalized against protein spots of the soluble and the insoluble protein fractions, respectively, which did not vary according to LNM state. In order to evaluate the significance of changes in LMAPs, a Student's *t*-test ($p \leq 0.05$) was performed.

MALDI-TOF-MS and database searching. For protein identification, spots of interest were excised from 2D-GE gels and destained with 50% acetonitrile in 0.1 M ammonium bicarbonate and dried in a SpeedVac evaporator (Savant, Farmingdale, NY, USA). Dried gel pieces were re-hydrated with 30 μl of 25 mM sodium bicarbonate, pH 8.8, containing 50 ng trypsin (Promega, Madison, WI, USA), at 37°C overnight. Samples were desalted using ZipTip C18 (Millipore, Bedford, MA, USA). Alpha-Cyano-4-hydroxycinnamic acid (10 mg) was dissolved in 1 ml of 50% acetonitrile in 0.1% trifluoroacetic acid and 1 μl of the matrix solution was mixed with an equivalent volume of sample. An analysis was performed using a 4700 Proteomics Analyzer TOF/TOF system (AB SCIEX, Foster City, CA, USA), which was operated in a positive ion reflection mode. Mass spectra were first calibrated in the closed external mode using a 4700 proteomics analyzer calibration mixture (AB SCIEX), and peptide mass peaks from each spectrum were submitted to the Mascot peptide mass fingerprinting search engine form (www.matrixscience.com) for analysis with GPS Explorer software, version 3.5 (AB SCIEX). The MS/MS spectra acquired were searched against Swiss-Prot and NCBI databases using an in-house version of MASCOT.

Liquid chromatography-MS. Proteins of interest were excised out of 2D-GE, destained with 50% acetonitrile in 0.1 M ammonium bicarbonate, and dried in a SpeedVac evaporator. Dried gel pieces were re-hydrated with 30 μl of 25 mM sodium bicarbonate at pH 8.8, containing 50 ng trypsin, at 37°C overnight. Samples were desalted using ZipTip C18, and dissolved in 10 μl of 2% acetonitrile in 0.1% formic acid. The analysis was performed using a LTQ-XL linear ion-trap mass spectrometer system (Thermo Scientific, Bremen, Germany). The MS was set for electrospray ionization in the positive mode. A syringe pump was used to introduce the calibration solution for automatic tuning and calibration of the LTQ spectrometer system in electrospray ionization positive-ion mode. Infusion of trypsin-digested 2D-GE samples into the ionization source of the MS was accomplished with liquid chromatographic separation. The spray voltage was set at +1.1 kV, while the temperature of the capillary was set at 200°C, the capillary voltage was set at +20 V and the tube lens voltage was set at +100 V. The auxiliary gas was set to zero. Full scan experiments were performed to a linear trap in the range of 150-2,000 m/z. Systematic MS/MS experiments were performed by changing the relative collision energy and monitoring the intensities of the fragment ions.

Immunoblotting. We validated the expression of identified proteins using western blots made from the same samples used for 2D-GE. Protein fractions were resolved by SDS polyacrylamide gel electrophoresis in a 10% tricine gel and blotted onto a 0.2 μm nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). Blocking with 5% non-fat milk was followed by overnight probing of the membranes at 4°C with a monoclonal antibody to vimentin, chaperonin 60 (HSP60), superoxide dismutase 2 (SOD2) and phosphatidylethanolamine binding protein 1 (PEBP1) at a dilution of 1/1000. A horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody (goat anti-mouse, dilution 1/10,000) was then applied for one hour followed by incubation with Amersham ECL Western Blotting detection reagents (GE Healthcare Limited, Buckinghamshire, United Kingdom) for 1 minute. A separate set of membranes was probed with a polyclonal, affinity-purified antibody at a dilution of 1/10,000 followed by another HRP-conjugated secondary antibody (goat anti-rabbit, dilution 1/12,500). Amersham Hyperfilm ECL film (GE Healthcare Limited) was then exposed for

Table I. *Differentially expressed proteins among BRAF mutation-harboring papillary thyroid carcinomas (PTCs) with differing lymph node metastasis (LNM) status.*

Spot no.		Protein name
Expression increased in PTCs without LNM	2	Calrecticulin
	3	Chaperonin 60
	8	Gelsolin
	15	Tropomycin alpha
	18	Cathepsin B
	24	Phosphatidylethanolamine binding protein 1
	25	Superoxide dismutase 2
	32	Triose-phosphate isomerase
	42	Lymphocyte activation gene-3 associated protein
	45	Laminin A
Expression decreased in PTCs without LNM	12	Aconitate hydratase, mitochondrial
	14	Vimentin

each membrane for the appropriate time. An anti-β-actin antibody was used as a loading control in this analysis.

The films were scanned on a flatbed GS-710 scanner (Bio-Rad, Hercules, CA, USA). Densitometry was carried out for monoclonal antibodies and anti-β-actin by using Quantity One 4.6 (Bio-Rad).

Immunohistochemical staining. Immunohistochemical staining was performed on 38 paraffin-embedded tissues from PTC cases, all carrying the *BRAF* mutation (19 cases had LNM and 19 cases did not). Staining was performed using a monoclonal antibody against vimentin (V9; Santa Cruz Biotechnology, Santa Cruz, CA, USA), an antibody against HSP60 (N20; Santa Cruz Biotechnology), an antibody against SOD2 (SOD2 RabMAb; Epitomics, Burlingame, CA, USA) and an antibody against PEBP1 (Rabbit Anti human PEBP1 PolyClonal antibody; Proteintech, Chicago, IL, USA). Paraffin sections were deparaffinized with xylene, rehydrated with a grade series of ethanol, and heated for 20 min in a microwave while immersed in citrate buffer (pH 6.0). The sections were then cooled at room temperature for 20 min. To block the endogenous hyperoxidase activity and biotin, the sections were incubated with 0.3% hydrogen peroxide in water for 10 min. The sections were then blocked in normal serum for 20 min followed by incubation with the primary antibodies for two hours at a dilution of 1/500, with the exception of PEBP1 which was used at 1/100. The percentage of positive cells examined was scored as 1 (1-10%), 2 (10-25%), 3 (25-75%), or 4 (>75%). The staining intensity was graded as 0 (negative), 1 (weaker than control), 2 (positive control), or 3 (stronger than control). If the percentage score was 1 and the intensity grade was 0 or 1, the protein was considered not to be expressed.

Association with clinicopathological characteristics. With the proteins validated by the techniques above, we compared the expression of the protein with known clinicopathological risk factors of PTC. For this comparison we constructed a test set of 121 random paraffin-embedded PTC samples.

Statistical analysis. STATA 10 (Stata Corporation, College Station, TX, USA) was used for statistical analysis. Differences between groups were analyzed using the Pearson's chi-square test. Results were considered significant if the *p*-value was less than 0.05.

Results

Detection of BRAF mutation. We found that four out of six frozen PTC tissues harbored the *BRAF* mutation by direct sequencing. Half of the patients whose cancer carried the *BRAF* mutation had LNM.

Protein identification. Differentially-expressed protein spots were observed among the four PTCs harboring the *BRAF* mutation according to the LNM status of their cases using 2D-GE (Figure 1). Ten protein spots had a higher signal in PTC samples from cases without LNM, whereas two protein spots had a lower signal. Proteins representing each spot were identified by MALDI-TOF-MS (Table I).

Immunoblotting. To confirm the expression of proteins identified by 2D-GE and MALDI-TOF-MS, we performed western blot analyses. Among the 12 proteins that showed differential expression, only four proteins showed consistent results on western blot analysis. Vimentin, HSP60, SOD2 and PEBP1 showed concordant results from the MALDI-TOF-MS after the 2D-GE and the western blot analysis. The expression of HSP60, SOD2 and PEBP1 was increased in PTC samples without LNM, while the one of vimentin was increased in PTC samples with LNM (Figure 2).

Immunohistochemical staining. To verify our western blot findings on tissues, we stained a validation set (38 PTC samples all harboring *BRAF* mutations; half from cases that had LNM) with antibodies against the four candidate proteins (Figure 3). As shown in Table II, only HSP60 expression differed significantly between samples from cases with LNM and those without. HSP60 protein expression was more frequent in PTC samples from cases without LNM than in cases with LNM (16/19 (84.2%) *vs.*
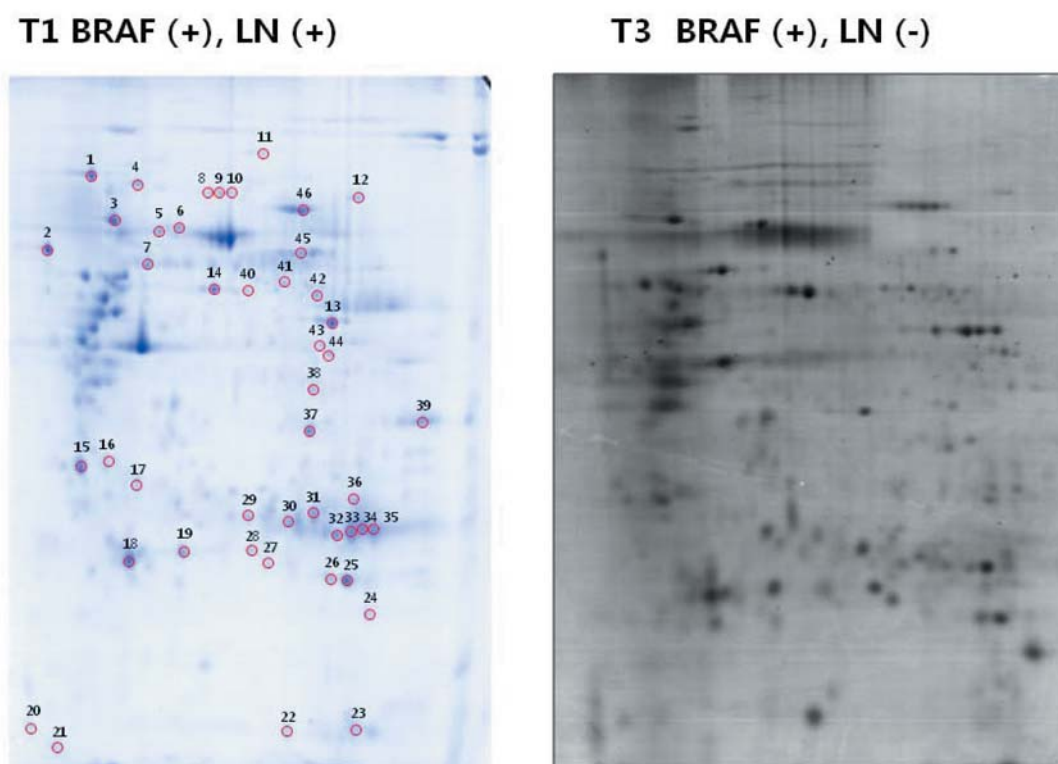
T1 BRAF (+), LN (+)   T3 BRAF (+), LN (-)

Figure 1. *Differential protein expression among papillary thyroid carcinomas harboring the BRAF mutation according to lymph node metastasis (LNM) status.*

Table II. *Results of immunohistochemistry and comparisons to lymph node metastasis (LNM) status in the validation set.*

LNM status	HSP60			SOD2		
	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Negative	3/19 (15.8%)	16/19 (84.2%)	0.003	5/19 (26.3%)	14/19 (73.7%)	0.248
Positive	12/19 (63.2%)	7/19 (36.8%)		8/19 (42.1%)	11/19 (57.9%)	
Overall	15/38 (39.5%)	23/38 (60.5%)		13/38 (34.2%)	25/38 (65.8%)	

	PEBP1			Vimentin		
	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Negative	7/19 (36.8%)	12/19 (63.2%)	0.364	4/19 (21.1%)	15/19 (78.9%)	0.330
Positive	5/19 (26.3%)	14/19 (737%)		2/19 (10.5%)	17/19 (89.5%)	
Overall	12/38 (31.6%)	26/38 (68.4%)		6/38 (15.8%)	22/38 (84.2%)	

HSP60: Chaperonin-60; SOD2: superoxide dismutase-2; PEBP1: Phosphatidylethanolamine binding protein-1.

Table III. *Demographics and tumor characteristics of the test set of 121 patients with papillary thyroid carcinoma.*

Parameter	No. (%)	Parameter	No. (%)
Demographics		Tumor characteristics	
Gender		Extrathyroidal invasion	
Male	16 (13.2)	Negative	42 (34.7)
Female	105 (86.8)	Positive	79 (65.3)
Age, years		Lymphovascular invasion	
≥45	79 (65.3)	Negative	94 (77.7)
<45	42 (34.7)	Positive	27 (22.3)
Stage		*BRAF* mutation	
T stage		Negative	23 (19.2)
T1	40 (33.1)	Positive	98 (81.8)
T2	4 (3.3)	Operation	
T3	77 (63.6)	Lobectomy	42 (34.7)
N stage		TT	67 (55.4)
N0	58 (47.9)	TT with MRND	12 (9.9)
N1a	48 (39.7)		
N1b	15 (12.4)		
Stage			
I	42 (34.7)		
II	19 (15.7)		
III	45 (37.2)		
IVa	15 (12.4)		

TT with MRND: Total thyroidectomy with modified radical neck dissection; *BRAF* mutation: T1799A *BRAF*[V600E] mutation.
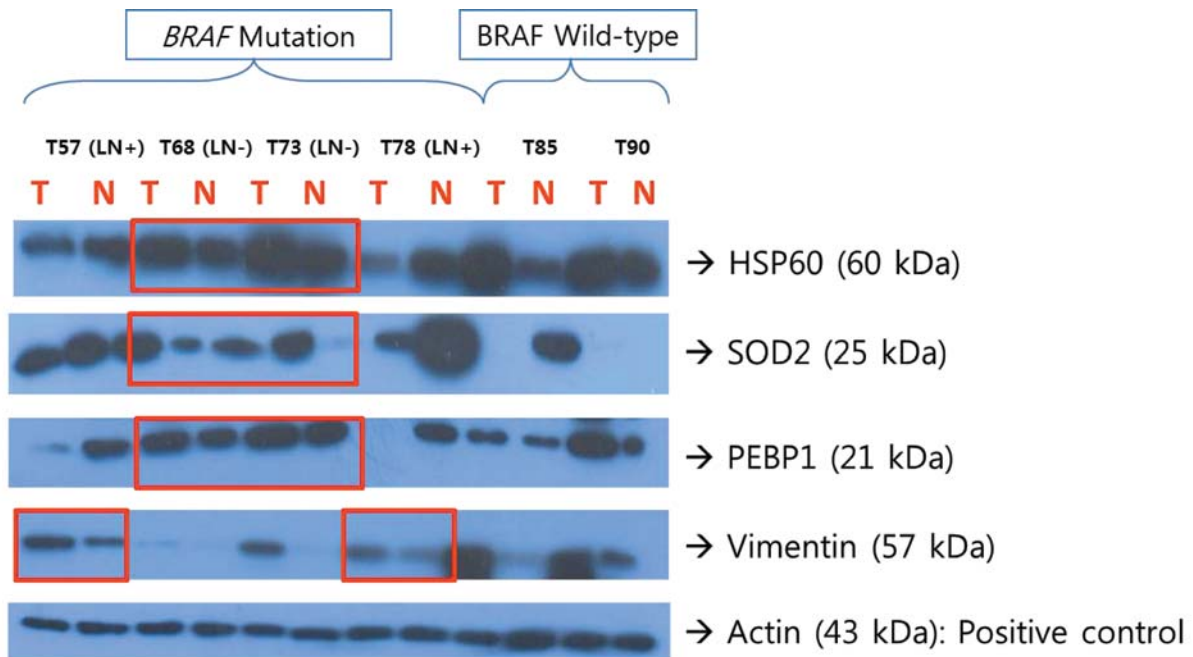
Figure 2. *Western blot results of proteins identified by MALDI-TOF-MS after 2D-GE. Expression of HSP60, SOD2 and PEBP1 increased in PTC samples without lymph node (LN) metastasis, while the of vimentin increased in PTC samples with LN metastasis. T=Tumor; N=normal.*
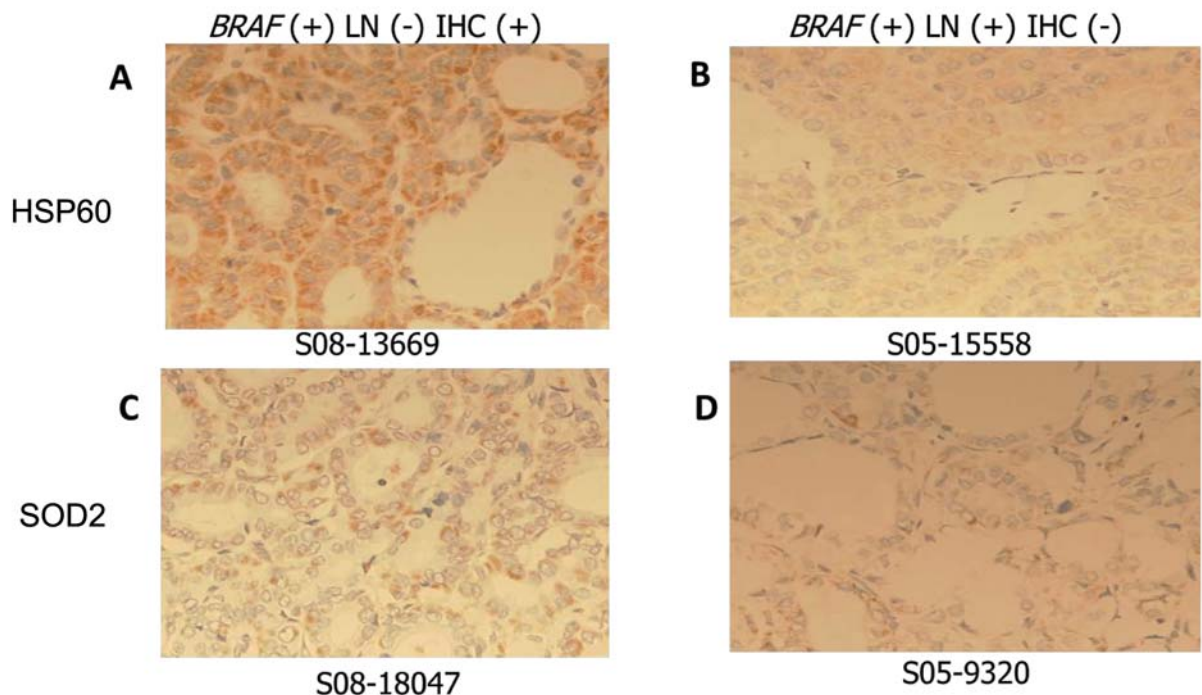

Figure 3. *Differences in immunohistochemical findings in the validation set according to lymph node (LN) metastasis. PTC samples without LN metastasis (A) and with LN metastasis (B) were immunohistochemically stained with an anti-HSP 60 antibody. (A) Was regarded as positive, but (B) was negative. PTC samples without LN metastasis (C) and with LN metastasis (D) were stained with an anti-SOD2 antibody; both were regarded as negative (original magnification ×400).*

Table IV. *Association between chaperonin-60 (HSP60) expression and lymph node metastasis (LNM) in the test set.*

BRAF mutation	HSP60	LNM		p-Value
		+	−	
		No. (%)	No. (%)	
Overall	Negative	5/21 (23.8)	16/21 (76.2)	0.015
	Positive	53/100 (53)	47/100 (47)	
Positive	Negative	4/19 (21.1)	15/19 (78.9)	0.003
	Positive	46/79 (58.2)	33/79 (41.8)	
Negative	Negative	1/2 (50.0)	1/2 (50.0)	0.585
	Positive	7/21 (33.3)	14/21 (66.7)	

7/19 (36.8%), $p=0.003$). Therefore, HSP60 appears to be a putative LMAP.

Other clinicopathological findings (such as tumor size, extrathyroid extension, demographics of patients) and certain protein expressions failed to exhibit significant differences (data not shown).

Association with clinicopathological characteristics. To assess the clinical significance of HSP60 as an LMAP, we compared the expression of HSP60 with traditional clinicopathological risk factors of PTC in a test sample set (Table III). HSP60 expression correlated with the absence of LNM solely in PTC samples harboring the *BRAF* mutation (Table IV).

Thus, HSP60 expression seems to be associated with inhibition of LNM in PTC cases harboring the *BRAF* mutation.

Discussion

The results of our study suggest that protein expression differs between PTCs with LNM and PTCs without LNM inspite of the presence of the *BRAF* mutation. Increased HSP60 expression may be associated with less frequent LNM.

HSP60 is considered extremely versatile because of its various effects on tumor cell survival (16). It has an essential role in tumor cell growth and is cytoprotective of tumor cells by increasing the apoptotic threshold for some malignancies (17, 18). HSP60 is also known for its ability to enhance caspase activation and then stimulate apoptosis (19, 20), however it can also stimulate an anti-apoptotic mechanism involving sequestration of a Bcl-2-associated X protein-containing complex (21). Thus, specific roles of HSP60 in tumor cell growth and proliferation are incompletely understood, but the monitoring of its expression levels and distribution in tissues can yield important information for diagnosis and prognosis assessment (22). Although HSP60 is considered an intra-mitochondrial protein, it has been found in the cytosol, on the surface of normal or tumor cells, and in the extracellular space (23-25).

Overexpression of HSP60 has been observed in astroglioma, acute myeloid leukemia, Hodgkin's lymphoma, cutaneous lichen planus, esophageal squamous cell cancer, gastric mucosa-associated lymphoid tissue lymphoma, colorectal carcinoma, hepatocellular carcinoma with hepatitis C virus-infected liver tissues, prostate cancer, cervical cancer, breast cancer, and adrenal Cushing tumor (26-36). On the contrary, in multiforme glioblastoma, small lymphocytic lymphoma, anaplastic large-cell lymphoma, immunoblastic lymphoma, bronchial adenocarcinoma, squamous cell carcinoma of the tongue, and vesical transitional-cell carcinoma, the levels of HSP60 are decreased (28, 37-40).

In addition, the relationship between HSP60 levels and prognosis is well-understood for some tumor types. In blasts of acute myeloid leukemia, HSP60 levels are elevated and correlate with a poor prognosis (27). High levels of HSP60 predict shorter overall survival and platinum-free interval of patients with serous ovarian carcinoma, since HSP60 mediates drug resistance (41). However, elevated levels of HSP60 are considered an indicator of good prognosis in esophageal squamous cell carcinoma, epithelial ovarian carcinoma, and vesical transitional-cell carcinoma (30, 42, 43).

To our knowledge, this is the first study that evaluated the association between HSP60 and PTCs through the use of proteomics. We found HSP60 protein expression may have roles in inhibiting LNM in PTCs, especially those harboring the *BRAF* mutation, although we did not determine the inhibiting mechanism. This finding suggests that HSP60 could be a good prognostic factor for patients with PTC.

Many findings from large-scale expression studies are required to clarify whether the changes observed derive from a sequential step or represent qualitative events

suggesting distinct processes. In this respect, application of proteomics seemed a feasible approach to comprehensively analyze gene and protein expression differences of thyroid tumors to identify missing pieces of the molecular puzzle (44). We aimed to identify prognostic biomarkers using a series of sequential proteomic experiments, consisting of a pilot study to raise clinical inquiry, efforts to identify differentially expressed proteins, more conventional experiments to validate our proteomic results, and a final attempt to identify clinical significance from a random patient population.

Acknowledgements

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