DNA Methylation of MAPK Signal-inhibiting Genes in Papillary Thyroid Carcinoma

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Abstract. *Background: The purpose of this study was to identify the DNA methylation status of the mitogen-activated protein kinase (MAPK) signal-inhibiting genes dual-specificity phosphatase 4 (DUSP4) and 6 (DUSP6); and serpin peptidase inhibitor A member 5 (SERPINA5) in thyroid cancer. Materials and Methods: Using 76 papillary thyroid cancer(PTC) tissues and three thyroid cancer cell lines (TPC1, WRO82-1 and XTC), the expression of three genes and DNA methylation were determined by reverse transcription-PCR and methylation-specific PCR. Results: In all cell lines, the expression of DUSP4 and DUSP6 increased; the corresponding gene promoters were unmethylated. However, SERPINA5 gene expression decreased and SERPINA5 DNA was methylated in the TPC1 cell line. With the de-methylating agent 5'-aza-2'-deoxycytidine, SERPINA5 gene expression was restored. In 82.9% of PTC tissues (63/76), the SERPINA5 DNA promoter was methylated, which was associated with a higher v-raf murine sarcoma viral oncogene homolog B1(BRAF) mutation rate in PTC tissues based on multivariate regression (odds ratio=3.573; 95% confidence interval=1.122-11.379; p=0.031). Conclusion: The expression of the MAPK signal-inhibiting gene SERPINA5 decreased in the TPC1 cell line, SERPINA5 expression was regulated by DNA methylation, which was associated with a higher BRAF mutation rate in PTC.*

The incidence of thyroid cancer is rapidly increasing (1). Although the prognosis of thyroid cancer is not grave, the recurrence rate was reported to be up to 30% (2), which worsens the quality of life of affected patients. Many factors were investigated in an effort to predict recurrence, but even the TNM stage could predict only mortality and not recurrence. A point-mutation in the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) gene reflects a high risk of recurrence (3). However, this mutation's prognostic power seems to be relatively limited in the iodine-sufficient countries in which *BRAF* mutation is prevalent.

Mitogen-activated protein kinase (MAPK) activation is modulated by negative feedback inhibition on multiple levels: at the receptor level (4), at the level of Rat sarcoma (RAS) or v-raf murine sarcoma viral oncogene (RAF) (5), and at the level of extracellular-signal-regulated kinases (ERK) by dual-specificity phosphatases (DUSPs). *BRAF* is a gene that is located at a high level of the MAPK signaling pathway and that controls cell growth and differentiation (6). If a V600E mutation develops in the *BRAF* gene, the MAPK pathway is constantly activated, which has been closely correlated with carcinogenesis, invasion and metastasis (7).

The actual biological behavior of *BRAF*-mutated tumors is regulated by other downstream molecules of the MAPK pathway, such as DUSP4, DUSP6 and serpin peptidase inhibitor A member 5 (SERPINA5). DUSP4, an ERK inhibitor, is expressed in nearly all tissues and encodes a phosphatase that inactivates MAPK. SERPINA5, a protein C inhibitor, also inhibits several plasma proteases involved in blood coagulation and is a potent inhibitor of urokinase (8). Urokinase is important for tumor cell metastasis, in which SERPINA5 acts as a potential tumor suppressor (9).

DNA methylation is a representative epigenetic mechanism that modulates gene expression. Because many tumor suppressor genes are suppressed in thyroid cancer (10, 11), we evaluated the DNA methylation profile of MAPK signal-inhibiting genes such as *DUSP4, DUSP6* and *SERPINA5* and the association between the methylation status and the expression of the genes. Furthermore, we elucidated the association between DNA methylation status and prognostic markers in papillary thyroid cancer (PTC).

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Key Words: Thyroid cancer, methylation, SERPINA5.

Materials and Methods

Patient samples. In total, 76 PTC tissue samples were obtained after total thyroidectomy with patients' consent; 10 tissues were obtained in a freshly-frozen state, and the other tissues were paraffin-embedded. In particular, the thyroid cancer specimens were matched with normal adjacent tissue in the 10 freshly-frozen cases. Clinical and histopathological data were reviewed for all of these patients (Table I). All tissue samples were collected using an Institutional Review Board-approved protocol at the National Cancer Center (NCCNCS-08-128).

Cell lines. The following thyroid cancer cell lines were studied: TPC1, WRO82-1 and XTC. The cells were kindly provided by Dr. Orlo H. Clark (University of California, San Francisco, CA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. All experiments were performed in RPMI-1640 (Gibco Laboratories) containing 10% serum (12).

mRNA preparation. Total RNA from the cells was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and an RNeasy kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction and 5 μM oligo(dT) primer (synthesized by Bioneer, Seoul, Korea).

DNA extraction from paraffin block and detection of BRAF mutation. The tumor areas were dissected from the formalin-fixed paraffin-embedded block (approximately 15-25 mg). Each sample was treated with xylene three times to remove the paraffin and with absolute ethanol to completely remove the xylene (Sigma, St. Louis, MO, USA). The sample was then digested with proteinase K (Sigma) for more than 24 h at 56˚C. DNA was isolated from the digested tissue using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA).

To detect for *BRAF* point-mutations, we performed direct sequencing in exon 15. The mutation rate of *BRAF* is known to be about 70%, and related to sufficient iodine intake of Korean population (13). The sequencing reactions were performed in an MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA).

PCR amplification. The PCR reaction buffers were mixed, consisting of 0.2 mM deoxynucleotide triphosphate, 1.5 U of Taq polymerase (Roche Molecular Biochemicals, Basel, Switzerland), 1 pmol of primer (Table II) and 1× PCR buffer solution. Amplification was performed with an automated thermal cycler (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 95˚C for 3 min followed by denaturation at 95˚C for 30 s, annealing at 56˚C for 30 s and elongation at 72˚C for 30 s for 35 cycles; and a final extension at 72˚C for 10 min. The PCR products were separated by electrophoresis on 1.8% agarose gels containing ethidium bromide.

Methylation-specific PCR analysis (MSP). For MSP analysis, 5 μg of genomic DNA was denatured in 0.4 M NaOH and modified with 2.5 M sodium bisulfite and 10 mM hydroquinone at 55˚C for 16 h.

Table I. *Clinicopathological characteristics of patients*.

Variable	*BRAF* mutation		Total
	No	Yes	
Age, years			
≥45	8/16	32/60	40/76
Gender			
Male	4/16	10/60	14/76
Surgery			
Lobectomy	1/16	4/60	5/76
Total thyroidectomy	15/16	56/60	71/76
T Stage			
1	7/16	24/60	3/76
2	1/16	0/60	1/76
3	8/16	36/60	44/76
N Stage			
0	11/16	27/60	38/76
1	5/16	33/60	38/76
Stage			
I	9/16	20/60	29/76
II	1/16	0/60	1/76
III	5/16	38/60	43/76
IV	1/16	2/60	3/76
Extrathyroidal extension	9/16	43/60	52/76
Tumor size, cm	1.20±0.66	1.40±0.67	
Number of metastatic LNs	2.75±4.66	2.68±4.74	

BRAF: v-raf murine sarcoma viral oncogene homolog B1, LN: lymph node.

After purification with a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA), the DNA was desulfonated in 3 M NaOH, precipitated in 100% ethanol and resuspended in distilled H_2O. Subsequently, 100 ng DNA was used as a template in the MSP reactions with 1.5 mM $MgCl_2$ and an adequate amount of primers specific for methylated and unmethylated DNA. The primers were designed to attach to the promoter region of each gene or the major transcription-factor binding site. The primer sequences and PCR conditions for MSP were listed in Table II.

Each reaction was tested with untreated DNA to ensure a lack of amplification, and two controls were included to ensure specificity: i) normal human fibroblast DNA previously treated with the CpG methylase SSS1 in the presence of S-adenosylmethionine (*in vitro*-methylated DNA), and ii) no template (blank). The PCR products were analyzed after electrophoresis on 4% agarose gels containing ethidium bromide. Direct sequencing of the amplified DNA was performed as a validation method to detect both methylated and unmethylated sequences.

5-aza-2'-deoxycytidine treatment of cell lines. All three cell lines (TPC1, WRO82-1 and XTC) were treated with 5-aza-2'-deoxycytidine (5'-aza; Sigma) in an attempt to demethylate the target genes. The cells were cultured in the above medium, to which 5'-aza was added to a concentration of 0.5 μM, 1 μM or 2.5 μM for 3 days before the extraction of DNA using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA).

Statistical analysis. To compare two groups, a Fisher's exact test and a Chi-square test were performed for categorical parameters,

and Mann-Whitney test for non-categorical parameters. To assess the relationship between *BRAF* and the methylation status of the target genes, univariate and multivariate logistic regression analyses were performed. All statistical analyses were performed using STATA9 (StataCorp LP, College Station, TX, USA). *p*-Values of less than 0.05 were considered statistically significant.

Results

mRNA levels and DNA methylation status of MAPK signal-inhibiting genes in thyroid cancer cells. The mRNA level of the MAPK signal-inhibiting genes *DUSP4*, *DUSP6* and *SERPINA5* and the changes in the level of mRNA expression after treatment with the demethylating agent 5'-aza were evaluated in three thyroid cancer cells (TPC1, WRO82-1 and XTC).

The mRNA level of *SERPINA5* varied in the three cell lines; *SERPINA5* expression was not detected in the TPC1 cells but was expressed in both the WRO82-1 and the XTC cell lines (Figure 1A). However, the expression of *DUSP4* and *DUSP6* was detected in all cell lines, and was not changed by 5'-aza treatment (Figure 1B and C). The response to 5'-aza treatment was also different between the cell types; the expression of *SERPINA5* was not changed in the XTC cells, whereas the expression of *SERPINA5* was restored in the TPC1 and WRO82-1 cell lines, with dose dependence in the TPC1 cell line. By MSP analysis, *SERPINA5* was found to be methylated in all three cell types but unmethylated after 5'-aza treatment (Figure 2). *DUSP4* and *DUSP6* were both unmethylated in the cells and not changed by 5'-aza treatment (data not shown). All MSP results were also validated by direct bisulfite sequencing (Figure 3).

DNA methylation status in normal and malignant thyroid tissues. DNA methylation status was also evaluated in 10 freshly-frozen PTC tissues by MSP analysis. *DUSP4* and *DUSP6* were unmethylated in both tumor and normal tissues. In contrast, *SERPINA5* was unmethylated in all normal tissues, except for three in which the methylation band was not visible, whereas *SERPINA5* was methylated in seven out of ten tumor tissues. The methylation patterns of representative cases are shown in Figure 4. Total expression of *SERPINA5* mRNA decreased in all tumors with methylated *SERPINA5*, whereas *SERPINA5* was detectable in its unmethylated form in both normal and tumor tissues.

Association between SERPINA5 DNA methylation and prognostic factors in PTC. We explored the DNA methylation status of *SERPINA5*, *DUSP4* and *DUSP6* in paraffin-embedded tissues to evaluate the association between DNA methylation status and alleged prognostic factors. *DUSP4* and *DUSP6* were both unmethylated in thyroid cancer tissue. However, *SERPINA5* was methylated in most tumors (63/76 cases, 83.9%).

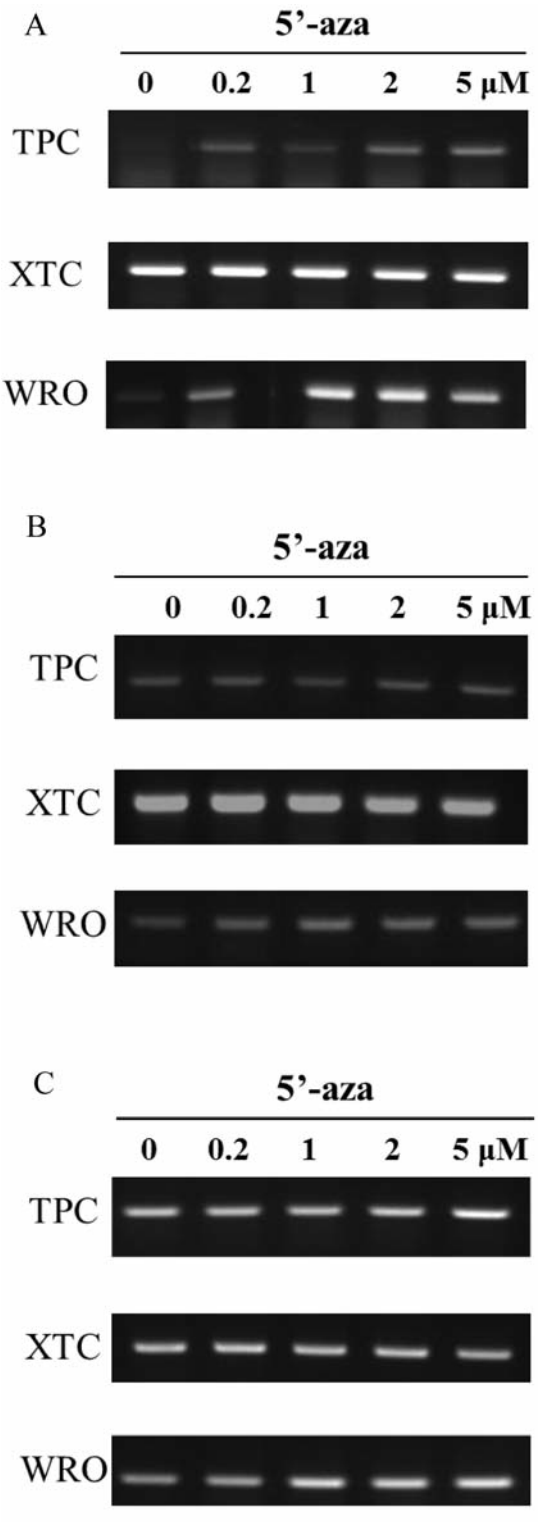

Figure 1. *Changes in the expression and methylation status of mitogen-activated protein kinase(MAPK)-inhibiting genes. A: serpin peptidase inhibitor A member 5(SERPINA5); B. dual-specificity phosphatase 4(DUSP4); and C. dual-specificity phosphatase 6 (DUSP6) after the administration of 5'-aza-2'-deoxycytidine (5'-aza) to thyroid cancer cell lines (TPC1, WRO82-1 and XTC).*

Table II. *Primer sequences used in PCR amplification and methylation-specific PCR analysis.*

		Primer sequence
DUSP6	Forward	CTT GGA CGT GTT GGA GGA AT
	Reverse	AAT GGC CTC AGG GAA AAA CT
DUSP4	Forward	AGG CGG CTA TGA GAG GTT TT
	Reverse	CAC TGC CGA GGT AGA GGA AG
SERPINA5	Forward	GTC CAG CAC AAA GAT GCA GA
	Reverse	CAG CGT CTT CAT GGC ACT TA
DUSP6 (methylated)	Forward	GTT TTA ATG ATT GAA ACG TTT C
	Reverse	ATT ACC GAA CTC CTC TCC G
DUSP6 (unmethylated)	Forward	AGG TTT TAA TGA TTG AAA TGT TTT
	Reverse	AAA TTA CCA AAC TCC TCT CCA
DUSP4 (methylated)	Forward	GTC GCG TTT TAT TTA AGT TTC G
	Reverse	AAA ATT TAT TAA TAC TCC TCC GCG
DUSP4 (unmethylated)	Forward	GGT TGT GTT TTA TTT AAG TTT TGG
	Reverse	AAA ATT TAT TAA TAC TCC TCC ACA CT
SERPINA5 (methylated)	Forward	GTT TGT AGG TAG GTT TGT TGG TC
	Reverse	ACA TCG ATA CTT ACT ATA TCC CGT A
SERPINA5 (unmethylated)	Forward	GTT TGT AGG TAG GTT TGT TGG TTG
	Reverse	ACA TCA ATA CTT ACT ATA TCC CAT A

DUSP4/6: Dual-specificity phosphatase 4 and 6, *SERPINA5*: serpin peptidase inhibitor A member 5.

Table III. *Univariate and multivariate analyses of serpin peptidase inhibitor A member 5(SERPINA5) promoter methylation as a risk factor for v-raf murine sarcoma viral oncogene homolog B1(BRAF) mutation.*

	Odds ratio	95% CI	*p*-Value
Chi-square	4.543	1.260-16.385	0.024
Univariate	5.300	2.697-10.417	<0.0001
Multivariate	3.573	1.122-11.379	0.031

CI: Confidence interval.

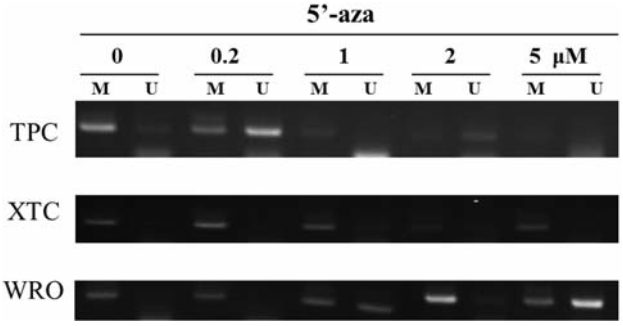

Figure 2. *Methylation-specific PCR analysis (MSP) comparison of serpin peptidase inhibitor A member 5 (SERPINA5) promoter methylation after 5'-aza-2'-deoxycytidine (5'-aza) treatment in thyroid cancer cell lines (TPC1, WRO82-1 and XTC). M: Methylated, U: unmethylated.*

Because *BRAF* is located in the MAPK signaling pathway and *SERPINA5* regulates certain enzymes that are downstream of the MAPK pathway, we first evaluated the association between the methylation status of *SERPINA5* and *BRAF* mutation, a genetic marker of poor prognosis in PTC. We found a significant positive correlation between methylation of *SERPINA5* and the *BRAF* V600E mutation (frequency of *BRAF* mutation: 60/76 overall, 7/13(53.8%) in tumors expressing unmethylated *SERPINA5 versus* 53/63(84.1%) in tumors expressing methylated *SERPINA5*, $p=0.025$). Logistic regression analysis confirmed the positive association between the *BRAF* mutation and *SERPINA5* promoter methylation (Table III). However, other clinicopathological factors, including lymph node (LN) metastasis, were not different between tumors expressing methylated *SERPINA5* and those expressing unmethylated *SERPINA5*, even according to *BRAF* mutation status (data not shown).

Discussion

We elucidated the expression of the MEK-ERK pathway inhibitory gene *SERPINA5*, which is regulated by the methylation of its DNA promoter. Additionally, we found that *SERPINA5* methylation is associated with *BRAF* mutation.

DNA methylation is one of many epigenetic regulatory mechanisms that silence a target gene by methylating the promoter region without affecting the genetic sequence itself (14, 15). In thyroid cancer cells, certain regulatory genes are silenced by DNA methylation, such as death-associated

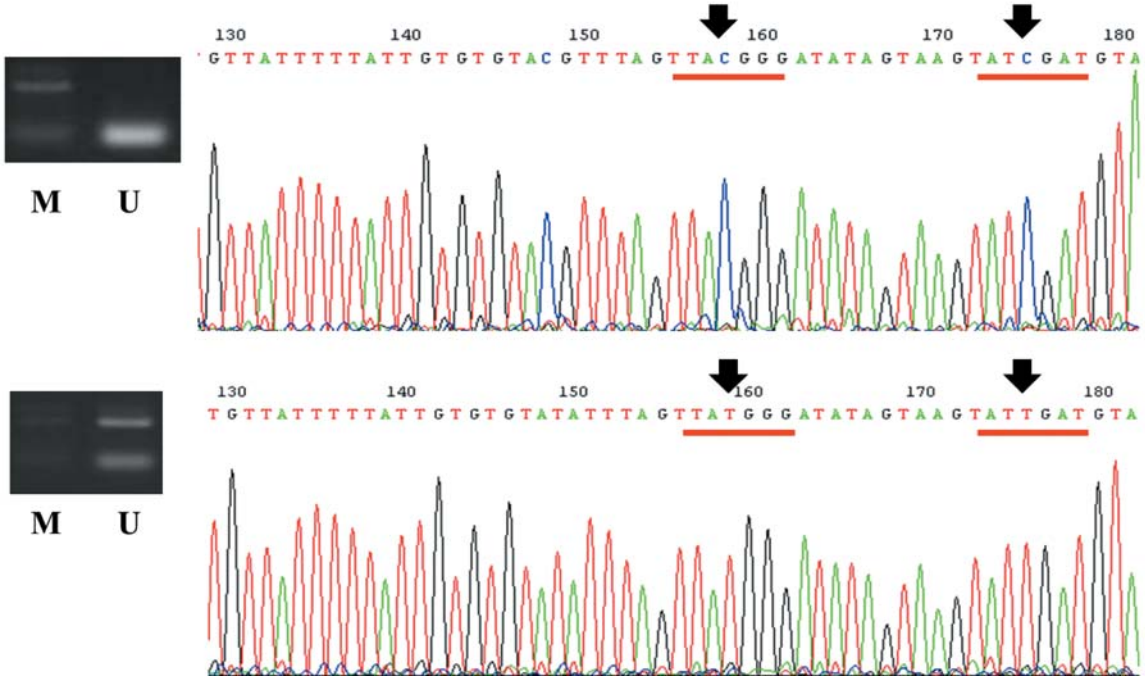

Figure 3. *Validation of Methylation-specific PCR analysis (MSP) for serpin peptidase inhibitor A member 5 (SERPINA5) by direct sequencing. M: Methylated, U: unmethylated. Black arrows: methylated loci.*

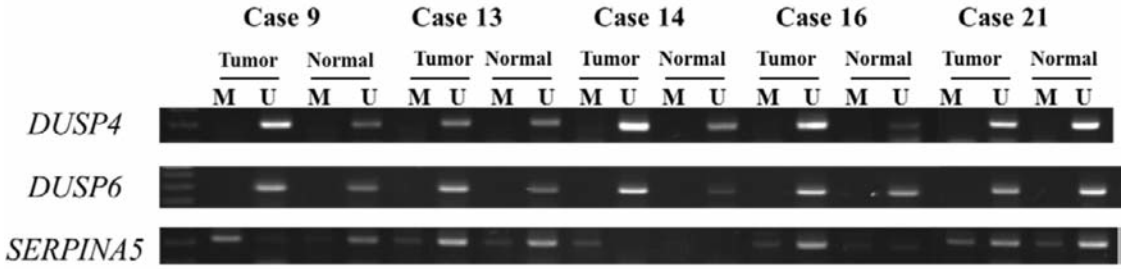

Figure 4. *Comparison of methylation profiles between tumor and normal tissues in representative cases. M: Methylated, U: unmethylated. DUSP4/6: dual-specificity phosphatase 4 and 6, SERPINA5: serpin peptidase inhibitor A member 5.*

protein kinase (DAPK), fibroblast growth factor receptor-2 (FGFR2), sodium iodide symporter (NIS), cyclin-dependent kinase inhibitor-2A (CDKN2A), Phosphatase and tensin homolog (PTEN), retinoic acid receptor B2 (RARB2), tissue inhibitor of metalloproteinases-3 (TIMP3) and thyroid stimulating hormone receptor (TSHR) (16-18). To our knowledge, we are the first to report the DNA methylation profiles of *DUSP4*, *DUSP6* and *SERPINA5* in thyroid cancer cell lines and tissues.

DUSPs are transcriptionally up-regulated by MAPK signaling *via* a negative feedback mechanism (19). These enzymes all display a high degree of substrate selectivity for ERK (20) and act as central feedback regulators attenuating ERK levels in developmental programs (21, 22). *DUSP4* localizes to the nucleus to dephosphorylate and thereby inactivate ERK, c-Jun N-terminal kinases (JNK) and p38 MAPKs (19). *DUSP6* determines the subcellular localization of ERK by serving as *a bona fide* cytoplasmic anchor for ERK, thereby mediating a spatiotemporal mechanism of ERK signaling regulation (23). The overexpression of *DUSP4* and *DUSP6* induces growth-suppressive effects in lung adenocarcinoma cell lines, with activating mutations of the MAPK pathways (23, 24). Our observation on the methylation status of *DUSP4* and *DUSP6* (unmethylated in thyroid cancer tissue) suggests that these tumor-suppressor genes do not actually suppress tumor growth.

SERPINA5 is a member of the superfamily of serine protease inhibitors including alpha-1-antichymotrypsin, alpha-1-antitrypsin, antithrombin III and angiotensinogen. The expression of *SERPINA5* is down-regulated in ovarian tumors with more aggressive behavior, which means that *SERPINA5* expression is a major indicator for the indolent behavior of borderline ovarian tumors (8). In thyroid cancer, the methylation of *TIMP3* is closely-associated with extrathyroidal invasion, LN metastasis and the multifocality of the tumor (10). In our study, *SERPINA5* promoter methylation was observed in 7/10 of PTC tissues, while all normal thyroid tissues were unmethylated.

The overexpression of *DUSP4* and *SERPINA5* was associated with the indolent behavior of *BRAF*-positive ovarian tumors due to suppression of matrix metalloproteinase (MMP9) activity (8). However, the role of these genes in *BRAF*-positive thyroid cancer has not yet been elucidated. We hypothesized that there would be a degree of cross-regulation between the *BRAF* mutation and the methylation of *SERPINA5* and examined the methylation profile of *SERPINA5* given *BRAF* mutation. Interestingly, the methylation status of *SERPINA5* was related to the frequency of *BRAF* mutation. The interim process has not been investigated yet; however, it may be an important indicator of what directs the change in the *SERPINA5* gene during the activation of the MAPK pathway. Recently, one genotyping study investigating immune-related single-nucleotide polymorphism (SNP)s showed that two SNPs (rs6115, rs6112) of *SERPINA5* were linked with the risk of PTC (25). The SNPs were also related with autoimmune thyroiditis, which might suggest that the methylation of *SERPINA5* is related with the relatively limited prognostic impact of *BRAF* mutations in PTC of patients in iodine-sufficient areas where both this mutation and autoimmune thyroiditis are frequent.

DUSP6 expression is up-regulated at the protein and mRNA levels in PTC and poorly-differentiated thyroid cancer, and *DUSP6* silencing reduces the neoplastic properties of PTC cell lines (26). We confirmed the elevated expression of *DUSP4* and *DUSP6* in thyroid cancer tissues, regardless of *BRAF* mutation (data not shown). This finding suggests that the activation of these two tumor suppressors is independent of *BRAF* mutation, which leads to the consistent activation of the upper-level molecule in the MAPK pathway.

MSP was limited to providing information at the level of individual CpG sites because there were various Cpg islands in the promoter areas of the candidate genes. However, we validated the results in randomly selected cases by direct sequencing. Additionally, the number of freshly-frozen tissues was small, which was another limitation of this study.

In summary, the expression of *SERPINA5* was reduced in PTC and regulated by DNA promoter methylation. *SERPINA5* methylation was associated with the *BRAF* mutation rate.

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Nothing to declare.

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