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-I 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).
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% CO2. 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), the DNA was desulfonated in 3 M NaOH, and precipitated in 100% ethanol and resuspended in distilled H2O. 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 H2O. Subsequently, 100 ng DNA was used as a template in the MSP reactions with 1.5 mM MgCl2 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.

**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 1x 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 bisulfitite and 10 mM hydroquinone at 55˚C for 16 h. 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 H2O. Subsequently, 100 ng DNA was used as a template in the MSP reactions with 1.5 mM MgCl2 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.

**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 \textit{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.

\section*{Results}

\textbf{mRNA levels and DNA methylation status of MAPK signal-inhibiting genes in thyroid cancer cells.} The mRNA level of the MAPK signal-inhibiting genes \textit{DUSP4}, \textit{DUSP6} and \textit{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 \textit{SERPINA5} varied in the three cell lines; \textit{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 \textit{DUSP4} and \textit{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 \textit{SERPINA5} was not changed in the XTC cells, whereas the expression of \textit{SERPINA5} was restored in the TPC1 and WRO82-1 cell lines, with dose dependence in the TPC1 cell line. By MSP analysis, \textit{SERPINA5} was found to be methylated in all three cell types but unmethylated after 5'-aza treatment (Figure 2). \textit{DUSP4} and \textit{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).

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

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

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{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).}
\end{figure}
Because \textit{BRAF} is located in the MAPK signaling pathway and \textit{SERPINA5} regulates certain enzymes that are downstream of the MAPK pathway, we first evaluated the association between the methylation status of \textit{SERPINA5} and \textit{BRAF} mutation, a genetic marker of poor prognosis in PTC. We found a significant positive correlation between methylation of \textit{SERPINA5} and the \textit{BRAF} V600E mutation (frequency of \textit{BRAF} mutation: 60/76 overall, 7/13(53.8\%) in tumors expressing unmethylated \textit{SERPINA5} versus 53/63(84.1\%) in tumors expressing methylated \textit{SERPINA5}, \textit{p}=0.025). Logistic regression analysis confirmed the positive association between the \textit{BRAF} mutation and \textit{SERPINA5} promoter methylation (Table III). However, other clinicopathological factors, including lymph node (LN) metastasis, were not different between tumors expressing methylated \textit{SERPINA5} and those expressing unmethylated \textit{SERPINA5}, even according to \textit{BRAF} mutation status (data not shown).

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

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\textit{DUSP4/6}: Dual-specificity phosphatase 4 and 6, \textit{SERPINA5}: serpin peptidase inhibitor A member 5.

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

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<td>Chi-square</td>
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CI: Confidence interval.

Discussion

We elucidated the expression of the MEK-ERK pathway inhibitory gene \textit{SERPINA5}, which is regulated by the methylation of its DNA promoter. Additionally, we found that \textit{SERPINA5} methylation is associated with \textit{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...
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 polymorphisms (SNPs) showed that two SNPs (rs61115, rs61112) 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.

Financial disclosure
Nothing to declare.

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Sidransky D: Correlation between BRAF mutation and promoter methylation of TIMP3, RARB2 and RASSF1A in thyroid cancer. Epigenetics 7(7): 710-719, 2012.


