Predictive Value of Sphingosine Kinase 1 Expression in Papillary Thyroid Carcinoma

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Abstract. Background/Aim: Sphingolipid metabolites are emerging as key signaling molecules in cancer. Sphingosine kinase 1 is up-regulated in many different types of human malignancies and plays a crucial role in cancer development and progression. The utility of sphingosine kinase 1 to act as a predictive biomarker in thyroid cancer remains unclear. Materials and Methods: Sphingosine kinase 1 expression was evaluated using immunohistochemical staining in 110 formalin-fixed, paraffin-embedded papillary thyroid carcinoma tissue samples. Results: Sphingosine kinase 1 expression in papillary thyroid carcinoma tissue was significantly higher than in nodular goiter (p<0.001) or normal thyroid (p<0.001) tissue. Sphingosine kinase 1 was observed in the cytoplasm of tumor cells. Thirty-four (30.9%) of 110 papillary thyroid carcinomas exhibited high sphingosine kinase 1 expression, that was significantly associated with tumor multiplicity (p=0.004), extrathyroidal extension (p=0.013), presence of lymph node metastasis (p<0.001), and number of metastatic lymph nodes (p=0.042). In addition, high sphingosine kinase 1 expression was the only independent predictor of lymph node metastasis (p<0.001). Conclusion: Sphingosine kinase 1 is involved in papillary thyroid carcinoma development and progression and can serve as a potential biomarker predictive of lymph node metastasis.

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Thyroid cancer is the most commonly diagnosed endocrine malignancy. Papillary thyroid carcinoma constitutes approximately 90% of all malignant thyroid neoplasms, generally with an indolent clinical course (1-3). Long-term outcomes of patients with papillary thyroid carcinoma are favorable, with a survival rate >90% (1). However, some papillary thyroid carcinoma cases display aggressive oncogenic behavior, and clinicopathological characteristics such as old age, large tumor size, extrathyroidal extension, and cervical lymph node metastasis have been suggested as factors for a poor prognosis (4-6). Similar to other human cancers, papillary thyroid carcinoma development, progression, and metastasis are caused by numerous genetic, reproductive, and environmental factors (3). Although many researchers have suggested potential biomarkers for papillary thyroid carcinoma to predict aggressive oncogenic behavior, no reliable biomarker has been identified. There is an urgent need to establish a predictive indicator to identify patients at high risk of metastasis and to enable clinicians to tailor therapeutic strategies to such patients and improve their outcomes (7).

Metastasis, a principal cause of cancer-related death, presents an important study target. In many human cancers, metastasis to the regional lymph node is one of the most significant predictors of patient outcome (8, 9). Lymph node metastasis is associated with decreased papillary thyroid carcinoma survival and increased risk of local recurrence. Outcome data from large institutional cohorts have shown a significant and independent negative impact of lymph node metastasis on outcomes in papillary thyroid carcinoma (10-12).

Sphingolipid metabolites are emerging as key signaling molecules in cancer (13, 14). Sphingolipid metabolism enzymes, sphingolipid-binding proteins, and transmembrane transporters of sphingolipid metabolites have important roles in cancer pathophysiology (15). Sphingosine kinases are important in cancer growth due to their ability to prevent apoptosis and stimulate cell proliferation and angiogenesis (14, 16). Two sphingosine kinase isoenzymes, sphingosine
kinase 1 and 2, have been identified in humans (17). Sphingosine 1-phosphate, a sphingolipid metabolite whose formation is catalyzed by sphingosine kinase 1, plays a crucial role in cell survival, growth, proliferation, and apoptosis (18).

Sphingosine kinase 1 is upregulated in cancers of the brain (19, 20), head and neck (18), colon (21, 22), stomach (16), ovaries (23), cervix (14), and lungs (24). Sphingosine kinase 1 is involved in cancer development, progression, metastasis, and neovascularization in the tumor microenvironment (25). However, data on sphingosine kinase 1 expression in thyroid cancer are limited. In this study, we analyzed the expression of sphingosine kinase 1 in papillary thyroid carcinoma tissue samples using a tissue microarray technique and immunohistochemical staining. In addition, we investigated the associations between sphingosine kinase 1 expression and clinicopathological characteristics of papillary thyroid carcinoma patients. Our observations suggest that sphingosine kinase 1 is involved in papillary thyroid carcinoma development and progression and can be useful as a predictive biomarker for lymph node metastasis.

Materials and Methods

Tissue specimens. This study (2017-08-017) was reviewed and approved by the Institutional Review Board of Kangbuk Samsung Hospital (Seoul, Republic of Korea). We selected 110 cases of papillary thyroid carcinoma and 16 cases of nodular goiter from the archival cases in the Kangbuk Samsung Hospital (Seoul, Republic of Korea). Eighty-one normal thyroid tissue samples were obtained from patients who had given informed consent and were used as controls. Tissues resected by surgeons were examined by pathologists before fixation in 10% neutral-buffered formalin. After fixation for 12-24 h, the tissues were thoroughly examined macroscopically and sectioned. After automatic tissue processing, sections were embedded in paraffin blocks. Four-micrometer-thick slices were cut from each formalin-fixed, paraffin-embedded tissue block using a rotary microtome, stained with hematoxylin and eosin, covered with a glass coverslip, and sent to two board-certified pathologists for examination and pathological diagnosis. Clinical and pathological information were obtained from the pathology reports as well as from electrical medical information systems. The information collected included age and sex of the patient, multiplicity and greatest dimension of tumor, presence of extrathyroidal extension and lymph node metastasis, number of metastatic lymph nodes, and BRAF mutational status.

Tissue microarray construction. Tissue microarray blocks were constructed as described previously (7). Briefly, all hematoxylin and eosin-stained slides were reviewed, and the two most representative tumor areas were marked on the corresponding formalin-fixed, paraffin-embedded tissue blocks. Two 2-mm-diameter tissue cores were obtained from each block and manually arrayed into recipient tissue microarray blocks. The assembly was held in an X-Y position guide with a 1-mm increment between individual cores, and the instrument was used to create holes in a recipient block with defined array cores. A needle was used to transfer the cores into the recipient block. The percentage of tumor volume in each core was >70%. Two tissue microarray blocks were prepared for each case.

Immunohistochemical staining. Immunohistochemical staining was performed using a compact polymer method (Bond Intense Detection Kit, Leica Biosystems, Newcastle upon Tyne, UK) (3, 7, 14, 26-30). The 4-μm-thick, formalin-fixed, paraffin-embedded slices were deparaffinized and dehydrated with xylene and then rehydrated in a graded series of alcohol solutions. Endogenous peroxidase activity was halted by incubation with 0.3% hydrogen peroxide and methanol for 20 min. Following a rinse in phosphate-buffered saline, slices were processed in citrate buffer (0.01 M, pH 6.0) and then irradiated in a microwave oven for 20 min and allowed to cool at room temperature. The primary antibody used was anti-SPHK1 antibody (1:100, polyclonal, Abgent, San Diego, CA, USA). After chromogenic visualization using a Dako Peroxidase/DAB EnVision+ Detection System (Dako, Agilent Technologies, Carpinteria, CA, USA), slices were counterstained with hematoxylin and coverslipped. Negative controls were stained without primary antibody.

Interpretation of immunohistochemical staining. The degree of sphingosine kinase 1 expression was evaluated by multiplying scores for the proportion of positively stained cancer cells and staining intensity (14, 20, 31). The proportion of stained cancer cells was scored as: 0, none; 1, 1-9%; 2, 10-49%; 3, ≥50% of all cancer cells. Staining intensity was scored as: 0, absent; 1, weak; 2, moderate; 3, strong. The final score was calculated as the product of proportion and intensity score, resulting in scores of 0, 1, 2, 3, 4, 6, and 9. The optimal cutoff value for high sphingosine kinase 1 expression was determined as described previously (14). A final score ≥4 was used to define high sphingosine kinase 1 expression. All slides were examined and scored by two board-certified pathologists who were blinded to the clinicopathological data and patient identity. Disagreements between the pathologists were resolved by consensus.

Detection of BRAF mutation. For detection of the BRAF V600E mutation, nucleic acids from fresh thyroid tissue were isolated using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) (3). Isolated nucleic acids were mixed with a polymerase chain reaction (PCR) master mix from a Seeplex BRAF V600E ACE Detection Kit (Seegene, Seoul, Republic of Korea). The mixed samples were immediately placed in a preheated thermal cycler for 15 min, and PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling amplification program consisted of 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 63°C, and extension for 1 min at 72°C. Amplified PCR products were loaded onto a 2% agarose gel and visualized with a SafeView Nucleic Acid Stain (Applied Biological Technologies, Carpinteria, CA, USA). The BRAF mutation was detected using a Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Chi-square or Fisher’s exact test was performed to compare the frequency of high sphingosine kinase 1 expression between papillary thyroid carcinoma, nodular goiter, and normal thyroid tissue and to analyze associations between sphingosine kinase 1 expression status and clinicopathological characteristics. Multivariate logistic regression analysis with a backward stepwise elimination method was used to identify independent predictors of lymph node metastasis. Statistical analyses were performed using PASW Statistics (version 18.0; IBM SPSS, Chicago, IL, USA). Statistical significance was defined as a p-value less than 0.05.
Results

Patient demographics. Patient age ranged from 23-73 years (median=43 years). Seventy-five (68.2%) of the 110 patients were women. The mean tumor size was 0.95 cm (range=0.2-5.5 cm), and 71 cases (64.5%) were diagnosed as microcarcinomas (<1.0 cm). Multifocal tumors and extrathyroidal tumor extension were noted in 26 (23.6%) and 55 (50.0%) cases, respectively. Lymph node metastases were identified in 64 (58.2%) patients. Thirty-three (51.6%) of 64 patients with lymph node metastases had three or more metastatic lymph nodes. BRAF mutational analysis was performed in 70 (63.6%) cases, 58 (82.9%) of which were positive.

Sphingosine kinase 1 expression. Representative photomicrographs of sphingosine kinase 1 expression in normal thyroid, nodular goiter, and papillary thyroid carcinoma tissue are shown in Figure 1. Sphingosine kinase 1 immunoreactivity was absent in all 81 normal thyroid tissues (Table I). No staining was observed in 12 (75.0%) of the 16 nodular goiter tissue samples, and the remaining four nodular goiter samples exhibited faint sphingosine kinase 1 expression in the cytoplasm of bland-appearing follicular epithelial cells. In contrast, papillary thyroid carcinoma tissues exhibited sphingosine kinase 1 expression with variable staining intensity and proportion. Thirty-four (30.9%) of the 110 papillary thyroid carcinoma tissue samples exhibited high sphingosine kinase 1 expression. Sphingosine kinase 1 expression was observed in the cytoplasm of tumor cells; a few tumor cells with strong cytoplasmic expression also displayed weak-to-moderate nuclear sphingosine kinase immunoreactivity. Frequency of high sphingosine kinase 1 expression in papillary thyroid carcinoma was significantly higher than in nodular goiter (p<0.001) or normal thyroid (p<0.001) tissue.

Sphingosine kinase 1 expression and clinicopathological characteristics of papillary thyroid carcinoma. Significant correlations were observed between high sphingosine kinase 1 expression and tumor multiplicity (p=0.004), extrathyroidal extension (p=0.013), lymph node metastasis (p<0.001), and number of metastatic lymph nodes (p=0.042) (Table II). There was a marginally significant association between sphingosine kinase 1 expression and the greatest dimension of the tumor (p=0.089). There was no significant association between sphingosine kinase 1 expression and patient age (p=0.547), sex (p=0.601), or BRAF mutational status (p=1.000).

Factors independently predicting lymph node metastasis in patients with papillary thyroid carcinoma. Tumor multiplicity...
Table I. Immunohistochemical expression of sphingosine kinase 1 in normal thyroid, nodular goiter, and papillary thyroid carcinoma tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Sphingosine kinase 1 expression, n (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High (%)</td>
<td>Low (%)</td>
<td></td>
</tr>
<tr>
<td>Normal thyroid tissue</td>
<td>81 0 (0.0)</td>
<td>81 (100.0)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Nodular goiter</td>
<td>16 0 (0.0)</td>
<td>81 (100.0)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Papillary thyroid carcinoma</td>
<td>110 34 (30.9)</td>
<td>76 (69.1)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant.

(\(p=0.027\)) and high sphingosine kinase 1 expression (\(p<0.001\)) were significantly associated with lymph node metastasis (Table III). Marginally significant correlations were observed between lymph node metastasis and sex (\(p=0.054\)) and extrathyroidal extension (\(p=0.053\)). To identify factors that independently predict lymph node metastasis, these four covariates were entered into multivariate logistic regression analysis. We found that high sphingosine kinase 1 expression was the only independent predictive factor for lymph node metastasis (\(p<0.001\), relative risk=6.907; 95% confidence interval=2.382-20.024). The remaining covariates – sex (\(p=0.064\)), tumor multiplicity (\(p=0.117\)), and extrathyroidal extension (\(p=0.227\)) – did not independently predict lymph node metastasis.

Discussion

In this study, we analyzed sphingosine kinase 1 expression in human papillary thyroid carcinoma. Sphingosine kinase 1 expression was significantly increased in thyroid cancer tissues compared to nodular goiter (\(p<0.001\)) and normal thyroid (\(p<0.001\)) tissues, consistent with previous results (14, 16, 20, 32-34). We observed that 34 (30.9%) of 110 papillary thyroid carcinoma tissue samples had high sphingosine kinase 1 expression. These findings indicate that sphingosine kinase 1 expression is up-regulated in papillary thyroid carcinoma and can be useful as a diagnostic biomarker for this type of carcinoma.

There have been only two previous studies of sphingosine kinase 1 expression in papillary thyroid carcinoma. Liang et al. (35) reported that overexpression of sphingosine kinase 1 differentially regulates expression of a number of microRNAs and mRNAs in the papillary thyroid carcinoma cell line, TPC1. They observed that sphingosine kinase 1 promoted cell invasion via interaction between miR-144-3p and fibronectin 1, suggesting a pro-invasive function. Guan et al. (31) reported that sphingosine kinase 1 expression in thyroid cancer is up-regulated and associated with expression of proliferating cell nuclear antigen and showed that silencing of sphingosine kinase 1 suppresses proliferation of thyroid cancer cells. Other studies have shown that increased sphingosine kinase 1 expression is associated with lymph node metastasis and distant metastasis of breast cancer (36). These findings are consistent with our data showing that high sphingosine kinase 1 expression is associated with aggressive oncogenic behavior including tumor multiplicity, extrathyroidal extension, and lymph node metastasis. Our results are also consistent with studies demonstrating an association between elevated sphingosine kinase 1 expression and aggressive oncogenic behavior such as larger tumor size, deeper invasion depth, advanced stage, worse histological differentiation, higher invasiveness, and chemotherapeutic resistance in cancers of the cervix (14), head and neck (37), thyroid (31), salivary duct (32), esophagus (38), colon/rectum (39), and bladder (40).

We observed that high sphingosine kinase 1 expression was the only independent predictor of lymph node
**Table III. Factors predictive of lymph node metastasis in patients with papillary thyroid carcinoma.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lymph node</td>
<td></td>
</tr>
<tr>
<td></td>
<td>metastasis</td>
<td>p-Value</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥43</td>
<td>25 (50.0)</td>
<td>0.112</td>
</tr>
<tr>
<td>&lt;43</td>
<td>39 (65.0)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>25 (71.4)</td>
<td>0.054</td>
</tr>
<tr>
<td>Woman</td>
<td>39 (52.0)</td>
<td>0.064</td>
</tr>
<tr>
<td>Multiplicity of tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>20 (76.9)</td>
<td>0.027*</td>
</tr>
<tr>
<td>Single</td>
<td>44 (52.4)</td>
<td>0.117</td>
</tr>
<tr>
<td>Greatest dimension of tumor (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1.0</td>
<td>25 (64.1)</td>
<td>0.351</td>
</tr>
<tr>
<td>&lt;1.0</td>
<td>39 (54.9)</td>
<td></td>
</tr>
<tr>
<td>Extrathyroidal extension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>37 (67.3)</td>
<td>0.053</td>
</tr>
<tr>
<td>Absent</td>
<td>27 (49.1)</td>
<td>0.227</td>
</tr>
<tr>
<td><strong>BRAF mutation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>33 (56.9)</td>
<td>0.662</td>
</tr>
<tr>
<td>Absent</td>
<td>6 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Sphingosine kinase 1 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>29 (85.3)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Low</td>
<td>35 (46.1)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant.

In conclusion, we demonstrated that sphingosine kinase 1 expression is significantly upregulated in papillary thyroid carcinoma and is associated with aggressive oncogenic behavior. High sphingosine kinase 1 expression was found to be an independent factor predictive of lymph node metastasis. Our results suggest that sphingosine kinase 1 promotes progression and metastasis of thyroid cancer and can serve as a predictive biomarker for lymph node metastasis.

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**References**


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