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Breast cancer sphingosine-1-phosphate is associated with phospho-sphingosine kinase 1 and lymphatic metastasis

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ABSTRACT

Background: Sphingosine-1-phosphate (S1P), a pleiotropic bioactive lipid mediator, has been implicated as a key regulatory molecule in cancer through its ability to promote cell proliferation, migration, angiogenesis, and lymphangiogenesis. Previous studies suggested that S1P produced by sphingosine kinase 1 (SphK1) in breast cancer plays important roles in progression of disease and metastasis. However, the associations between S1P and clinical parameters in human breast cancer have not been well investigated to date.

Materials and methods: We determined levels of S1P and other sphingolipids in breast cancer tissue by electrospray ionization-tandem mass spectrometry. Associations between S1P levels and clinicopathologic features of the tumors were analyzed. Expression of phospho-SphK1 (pSphK1) in breast cancer tissues was determined by immunohistochemical scoring.

Results: Levels of S1P in breast cancer tissues were significantly higher in patients with high white blood cell count in the blood than those patients without. S1P levels were lower in patients with human epidermal growth factor receptor 2 overexpression and/or amplification than those patients without. Furthermore, cancer tissues with high pSphK1 expression showed significantly higher levels of S1P than cancer tissues without. Finally, patients with lymph node metastasis showed significantly higher levels of S1P in tumor tissues than the patients with negative nodes.

Conclusions: To our knowledge, this is the first study to demonstrate that high expression of pSphK1 is associated with higher levels of S1P, which in turn is associated with lymphatic metastasis in breast cancer.

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Introduction

Breast cancer is the most common cancer diagnosis, and the second most common cause of cancer death among women in the United States.[1] Lymph node metastasis is a hallmark of breast cancer and is one of the major determinants of clinical staging and prognosis.[2] There has been growing evidence that tumor lymphangiogenesis, formation of new lymphatic vessels from the preexisting ones, plays an important role during lymphatic metastasis.[3-5] A number of signaling proteins, such as vascular endothelial growth factor -C/D and angiopoietins, are reported to mediate lymphangiogenesis. In addition to these signaling proteins, it has been recently discovered that bioactive lipid mediators also play critical roles in lymphangiogenesis and lymph node metastasis, thus providing new insights into the mechanisms of cancer metastasis.[6]

Sphingosine-1-phosphate (S1P), a bioactive lipid mediator, has been implicated as a key regulatory molecule in cancer through its ability to promote cell proliferation, migration, invasion, angiogenesis, and lymphangiogenesis.[7-12] S1P is generated intracellular by two sphingosine kinases, sphingosine kinase 1 and 2 (SphK1 and SphK2), and it is exported out of the cells where it regulates many functions by binding to and signaling through a family of five G–protein-coupled receptors (S1PR1–5).[8,13] This process, known as "inside-out" signaling, explains the autocrine and paracrine actions of S1P.[13] In breast cancer, we have previously demonstrated that SphK1, but not SphK2, is involved in S1P export from breast cancer cells through the action of adenosine triphosphate-binding cassette transporters, ABCC1 and ABCG2.[14-16] It has been demonstrated that SphK1 mRNA expression is upregulated in breast cancer, correlates with poor prognosis, and is associated with resistance to chemotherapy.[17-19] These studies suggest that S1P plays important roles in cancer progression and metastasis. However, the actual quantification of S1P in human breast cancer tissues has not been well investigated to date, limiting its ability to be used clinically as a biomarker. Furthermore, quantification of S1P mass in breast cancer could allow more accurate comparisons and evaluation of function between studies.

The aim of this study is to determine the mass quantities of bioactive sphingolipids, including sphingosine, S1P, dihydrosphingosine, and dihydro-S1P, in human patient breast cancer tissue samples using high sensitivity liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and to investigate associations between the levels of sphingolipids and clinicopathologic features of the tumor. Furthermore, we hypothesized that activation of SphK1 is associated with higher levels of S1P in tumor. To test this hypothesis, we performed immunohistochemical staining of active form of phospho-SphK1 (pSphK1) and compared it with the S1P levels in tumor.

Material and methods

Human breast cancer tissue samples

From January 2013 to April 2015, 289 Japanese patients with breast cancer underwent surgical resection in Niigata University Medical and Dental Hospital. During this period, breast cancer tissue samples were collected from 47 patients who had invasive tumors larger than 1.5 cm after obtaining informed consent. We analyzed 35 of the 47 primary breast cancer tissue samples, excluding a total of 12 patients. The excluded tissue samples included five patients who received neoadjuvant chemotherapy, two patients with body mass index greater than 35, three patients with bilateral breast cancer, and four patients with cancer in other organs. These patients were excluded because the above factors in these patients possibly affect levels of S1P in the body.[20] The blood counts were measured as a part of preoperative work-up, which is within 30 days before the operation. All tissue samples were snap-frozen and stored at −80°C. This study protocol was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital.

Quantification of sphingolipids by LC-ESI-MS/MS

Lipids were extracted from tissue samples, and sphingolipids were quantified by LC-ESI-MS/MS (4000 QTRAP, ABI) at the Virginia Commonwealth University Lipidomics Core as described previously.[6,15,21] Internal standards were purchased from Avanti Polar Lipids (Alabaster, AL) and added to samples in 20 μL ethanol:methanol:water (7:2:1) in a cocktail of 500 pmol each. The high performance liquid chromatography grade solvents were obtained from VWR (West Chester, PA).

Pathologic examination

All of the human breast cancer tissue specimens were submitted to the Department of Surgical Pathology in our hospital and examined by two experienced pathologists who had no access to the clinical data. Paraffin-embedded blocks from each resected specimen were used for immunohistochemistry. Serial 4-μm sections were recut and used for staining with hematoxylin and eosin, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), Ki-67–proliferation index marker, pSphK1, and negative control. The ER- and PgR-protein expression was scored using the weighted Allred score technique. Ki-67 index was scored by counting positive and negative nuclei in the tumor specimen, and a proliferation index was obtained by calculating the percentage of positive cells. Tumors were classified as having low or high proliferation index with 14% positive cells as the cutoff. HER2 expression was also determined by immunohistochemistry and by fluorescence *in situ* hybridization analysis. HER2 positive tumors were defined as 3+ on immunohistochemistry or as positive by fluorescence *in situ* hybridization.

Antigen retrieval for pSphK1 was performed by microwaving the slides under pressure in a citrate buffer for 10 min (pH 9.0). Endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 20 min. After blocking nonspecific background, the sections were incubated overnight with the primary antibody (SphK1 polyclonal antibody; 1:100 dilution; ECM Biosciences LLC, Versailles, KY) at 4°C. Then, the sections were incubated with biotinylated rabbit