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High levels of sphingolipids in human breast cancer

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Abstract

Background—Sphingolipids, including sphingosine-1-phosphate (S1P) and ceramide, have emerged as key regulatory molecules that control various aspects of cell growth and proliferation in cancer. Although important roles of sphingolipids in breast cancer progression have been reported in experimental models, their roles in human patients have yet to be determined. The aim of this study is to determine the levels of sphingolipids including S1P, ceramides, and other sphingolipids, in breast cancer and normal breast tissue and to compare the difference in levels of each sphingolipid between the two tissues.

Materials and Methods—Tumor and non-cancerous breast tissue were obtained from 12 patients with breast cancer. Sphingolipids including S1P, ceramides, and their metabolites of sphingosine, sphingomyelin, and monohexosylceramide were measured by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

Results—The levels of S1P, ceramides, and other sphingolipids in the tumor were significantly higher than those in normal breast tissue. There was a relatively strong correlation in the levels of S1P between the tumor and that of normal breast tissue from the same person. On the other hand, there was no correlation in the levels of most of the ceramide species between the tumor and that of normal breast tissue from the same person.

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Author contributions

M.N. conceived the study. M.N., J.T., K.M., M.H., and K.T. carried out experiments. M.N. and T.K. wrote the manuscript with assistance from I.A.W. and T.W.

Disclosure

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Conclusions—To our knowledge this is the first study to reveal that levels of sphingolipids in cancer tissue are generally higher than normal breast tissue in patients with breast cancer. The correlation of S1P levels in these tissues implicates the role of S1P in interaction between cancer and the tumor microenvironment.

Keywords

Sphingosine-1-phosphate; Ceramides; Breast cancer; Tumor microenvironment

1. 1. Introduction

Recent advances in molecular biology have provided new treatment strategies for breast cancer patients with molecular based targeted therapies. These have the capacity to regulate critical molecular mechanisms of breast cancer growth such as cell cycle, cell survival, DNA double strand breaks repair or tumor immunology [1–4]. Despite these advances in breast cancer therapy, approximately 40,000 women still die from breast cancer every year in the United States and breast cancer remains the leading cause of cancer related death for women aged 20 to 59 years in the world [5]. Deepening our understanding of the biology of breast cancer progression is key to developing better treatment strategies and achieving long-lasting therapeutic efficacies against breast cancer [6].

There has been increasing evidence that lipid mediators play pivotal roles in cancer biology and the disease process [7–9]. Cancer progression requires the capacity for cell growth, invasion, angiogenesis and metastasis. [10–12]. Bioactive sphingolipids, such as sphingosine-1-phosphate (S1P) and ceramides, have been emerging as important signaling molecules that regulate these critical processes [7–9]. Despite the importance of these lipid mediators in cancer biology, it often had been “overlooked”, since proteins, but not lipids, have been considered the primary signaling molecules involved in cancer progression. Moreover, unlike protein, it had been difficult to measure the absolute amount of sphingolipids in tissue samples due to a lack of technology until recently. Because of these reasons, the importance of lipid mediators on cancer biology in human has not been fully evaluated.

S1P is generated from sphingosine (Sph) by sphingosine kinases inside the cell and transported to the extracellular space by dedicated proteins in the cell membrane [13, 14]. Once outside, it exerts many biological functions by stimulating its specific G-protein coupled receptors (S1PR1-5) [15]. S1P is involved in numerous cellular functions such as cell proliferation, migration, survival, immune cell trafficking, angiogenesis and lymphangiogenesis, all of which are related to cancer progression and metastasis [16–19].

On the other hand, ceramide has been reported to mediate anti-proliferative responses, such as cell growth inhibition, apoptosis induction, senescence modulation and autophagy. Interestingly, recent studies suggest that de novo-generated ceramides may have distinct functions based on their fatty acid chain lengths, subcellular localization and direct downstream targets [16, 20]. Ceramide levels increase after treatment of cells with diverse apoptosis-inducing agents such as ionizing radiations, UV light, TNF- α and chemotherapeutic agents [20]. In breast cancer and normal cells ceramides can be generated

from Sph, sphingomyelin (SM), and monohexosylceramide (HexCer) [21]. It has been reported that the most abundant sphingolipid is SM, followed by HexCer and ceramide in normal cells [21]. Therefore, it is important to measure levels of SM, HexCer, and ceramide to investigate the role of sphingolipids in cancer.

The vast majority of the studies on sphingolipids, including S1P and ceramides in cancer, have utilized *in vitro* and *in vivo* experimental systems [22]. Although important roles of sphingolipids in breast cancer progression have been published numerous times in experimental models, the evidence in human tissue and patients is very limited. Importantly, there has been no study to determine accurate levels of sphingolipids in breast cancer and normal breast tissue due to a lack of technology to measure the sphingolipids. Recently, advances in mass spectrometry allow us to determine the amount of sphingolipids in human samples accurately [15, 23, 24].

The aim of this study is to determine the levels of bioactive sphingolipids (S1P, ceramides) and their metabolites (Sph, SM, and HexCer) in breast cancer and normal breast tissue and to elucidate the difference in levels of each sphingolipid between the two tissues. To our knowledge, this is the first study to reveal that levels of sphingolipids in human breast cancer tissue are generally higher than normal breast tissue. Further understanding the underlying mechanisms of increased levels of sphingolipids in breast cancer tissue in human will deepen our knowledge of cancer biology and provide a foundation to develop new therapeutic strategies to fight breast cancer.

1. 2. Material and Methods

1. 2.1. Tissue samples

Cancer tissue was collected from 12 patients who underwent mastectomy with invasive cancer larger than 1.5 cm. Subtypes of the patients are shown in Supplementary Table 1. All the patients agreed to provide the tissue for this study with informed consent. Matched normal breast tissue was collected in the most distant area from the tumor in each surgical specimen. 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.

2.2. Quantitation of Sphingolipids by LC-ESI-MS/MS

Internal standards were purchased from Avanti Polar Lipids (Alabaster, AL) and added to samples in 20 μl ethanol:methanol:water (7:2:1) as a cocktail of 500 pmol each. The HPLC grade solvents were obtained from VWR (West Chester, PA). Lipids were extracted from tissue samples and sphingolipids were quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESIMS/MS, 4000 QTRAP, ABI) as described previously at Virginia Commonwealth University Lipidomics Core [13, 23].

2.3. Statistical Analysis

All statistical evaluations were performed using the SPSS 22.0J software package (SPSS Japan, Tokyo, Japan). Categorical variables were compared by the Fisher exact test or the

Pearson χ^2 test and continuous variables between two groups were compared by the Mann-Whitney U test. All tests were two-sided and P values < 0.05 were considered statistically significant. Correlation between levels of sphingolipids in breast cancer tissue and those in normal breast tissue are denoted by R^2 . $R^2 \geq 0.81$ is considered a large linear association and $R^2 \geq 0.20$ is considered a small linear association.

3. Results

3.1. Determination of sphingosine-1-phosphate (S1P) and other sphingolipid levels in breast cancer and normal breast tissue

The aim of this study is to determine the levels of bioactive sphingolipids in human breast cancer and normal breast tissue. We successfully detected mass levels of Sph, dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in breast cancer and normal breast tissue (Fig. 1). All of these sphingolipids levels were universally higher in the cancer tissue than in the normal breast tissue (Fig. 1). There are relatively strong correlations in the levels of S1P and DHS1P between breast cancer and normal breast tissue ($R^2 = 0.7326, 0.7962$, respectively, Fig. 2). The levels of DHSph also show a weak correlation between breast cancer and normal breast tissue, while there was no correlation between the levels of Sph in the breast cancer and the normal breast tissue (Fig. 2).

3.2. Determination of sphingomyelin (SM) levels in breast cancer and normal breast tissue

We next determined the levels of each species of SM (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1 and C26:0) in the breast cancer and normal breast tissue (Fig. 3). Similar to the S1P and DHS1P, all of the species of SM levels were significantly higher in breast cancer than in the normal breast tissue (Fig. 3). There is a strong correlation in the levels of C18:1 SM between breast cancer and normal breast tissue ($R^2 = 0.8465$, Fig. 4). The levels of all other species of SM, except C14:0, display a correlation between breast cancer and normal breast tissue (Fig. 4).

3.3. Determination of HexCer levels in breast cancer and normal breast tissue

The levels of each species of HexCer (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1 and C26:0) in breast cancer and normal breast tissue were determined (Fig. 5). Similar to the SM, most of the levels of species of HexCer, except C14:0 and C18:1 HexCer, were significantly higher in the cancer tissue than in the normal breast tissue (Fig. 5). There are strong correlations between the levels of C14:0, C18:1, and C18:0 HexCer ($R^2 = 0.8461, 0.8988$, and 0.9373 respectively, Fig. 6). Although weaker, all the other HexCer species also demonstrated a correlation between breast cancer and normal breast tissue (Fig. 6).

3.4. Determination of ceramides levels in breast cancer and normal breast tissue

Finally, the levels of each species of ceramide (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1 and C26:0) in breast cancer and normal breast tissue were determined (Fig. 7). Similar to the other sphingolipids, most of the species of ceramide levels were significantly higher in the cancer tissue than in the normal breast tissue, except the C14:0, C18:1, and C26:0 ceramides. (Fig. 7). Interestingly, there is no correlation in the levels of most of the ceramide species between the breast cancer and normal breast tissue (Fig. 8).

The only exceptions were C14:0 and C18:0 ceramides, which did show a correlation between the breast cancer and normal breast tissue ($R^2 = 0.7195$ and 0.2371 , respectively, Fig. 8).

1. 4. Discussion

Although bioactive sphingolipids have attracted increasing attention as important mediators in cancer progression [7, 16], it had been difficult to measure the actual levels of sphingolipids until recently due to the lack of methods to determine them. Advances in lipidomics utilizing mass spectrometry allow us to determine exact levels of sphingolipids, including S1P, Sph, SM, HexCer, and ceramides, in tissue from human patients. It has been reported that the levels of ceramide in the cancerous tissue were higher than that of normal tissue [25]. However, there is currently no publication that has determined the exact levels of S1P and other important sphingolipids in breast cancer. Neither is there a publication that compares cancerous and normal tissue from the same patients. To our knowledge, this is the first study to quantify the levels of sphingolipids both in breast cancer and in normal breast tissue and to reveal the correlation of the levels of sphingolipids in the two tissues. We sought to further define the importance of the role of sphingolipids in cancer biology in this way.

In this study, we demonstrated that mass levels of Sph, DHSph, S1P, and DHS1P are higher in breast cancer than in normal breast tissue. We have previously reported that cancer cells express higher levels of SphK1 than normal mammary glands in animal models and S1P levels in tumor tissue are higher than normal mammary glands [24]. We also previously demonstrated that S1P, produced by SphK1, is a crucial mediator of breast cancer-induced angiogenesis, lymphangiogenesis and promotion of metastasis [24]. There have been increasing numbers of clinical and pathological reports that reveal the importance of SphK1 on metastasis and the prognosis of the cancer patients, however no quantified determination of S1P levels has been established [26–30]. During this study we found that the levels of S1P are higher in the breast cancer compared to the normal breast tissue, in agreement with the previous reports of higher expression of SphK1 in the human breast cancer. Together with our previous report that the serum S1P levels from patients with lymph node metastasis are higher than those from age and ethnicity-matched healthy volunteers [24], our findings further support the notion that S1P plays an important role in breast cancer progression.

We found correlations in S1P levels between the breast cancer and the normal breast tissue. It has been reported that blood and lymphatic endothelial cells in addition to inflammatory cells such as lymphocytes and macrophages, also strongly express SphK1, which produces S1P [18, 31, 32]. Therefore it is possible that factors produced by the cancer cells, such as IL-6 and TNF-alpha, and extruded into the tumor microenvironment induce the noncancerous host cells to produce S1P [18]. Given the fact that S1P is highly produced in cancer tissue and also in the surrounding normal breast tissue we cannot help but to speculate that S1P is not only produced in the cancer cells themselves but is also produced by host cells in the tumor microenvironment. It has been reported that levels of S1P in circulation can be affected by chronic inflammatory systemic diseases such as obesity, diabetes mellitus, or cardiovascular diseases [33, 34]. Taken together, the correlation of

increased S1P levels in cancerous tissue and the normal breast tissue is evidence of the delicate interaction between the cancerous cells and the host tumor microenvironment.

SM and HexCer were also measured in this study and both of these sphingolipids are higher in the cancer tissue compared to the normal breast tissue. There also were correlations between the levels of these lipids in these tissues. Although SM and HexCer have been understudied compared to S1P and ceramides, our findings suggest that the interaction between cancer and the tumor microenvironment affect the production of SM and HexCer. Further studies are needed to elucidate the impact of these lipids in breast cancer progression and biology.

Similar to the sphingolipids described above, most of the species of ceramide had levels that were significantly higher in cancer than in the normal breast tissue. This is in agreement with previous reports [25]. Interestingly, we found that there is no correlation in the levels of most of the ceramide species between breast cancer and normal breast tissue in contrast to the correlations found in levels of S1P, SM, and HexCer. It has been reported that ceramides play important roles in cancer biology, especially cell survival and death by regulating apoptosis. Since apoptotic cells that release intracellular ceramides at cell lysis are considered the major source of ceramides in the cancer tissue, the production of ceramides appears to occur independently from the condition of the host. Further studies are needed to investigate the role of ceramide in breast cancer and its tumor microenvironment.

In the current study, we demonstrated that levels of sphingolipids in breast cancer tissue are generally higher than normal breast tissue, which suggests that sphingolipids play important roles in breast cancer progression in human patients. Interaction between cancer cells and their tumor microenvironment is now considered to be a key to understanding cancer biology. Considering that S1P and other sphingolipids act as mediators inside and outside of cancer cells to promote their progression, it appears that sphingolipids play a pivotal role in the tumor microenvironment. Utilizing advanced technologies, such as matrix-assisted laser desorption and ionization (MALDI) mass spectrometry, we will be able to investigate the precise distribution of the sphingolipids on the cancer and surrounding normal tissue in surgical specimens [35]. It will also be important to determine the levels of sphingolipids in the interstitial fluid of the tumor. This fluid bathes the cancer and stromal cells and is considered an important part of the tumor microenvironment. The methodology to determine the levels of sphingolipids in the interstitial fluid of the tumor is currently under development. Ultimately, utilizing these cutting edge technologies to increase our understanding of the function of sphingolipids in cancer progression will provide us better strategies to target these lipids for cancer therapeutics.

1. 5. Conclusions

To our knowledge this is the first study to reveal that levels of sphingolipids in breast cancer are generally higher than normal breast tissue. The correlation of S1P levels in these tissues implicates the role of S1P in interactions between cancer and the tumor microenvironment. Our results indicate the importance of the role of sphingolipids in cancer biology in human patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Levels of sphingosine-1-phosphate (S1P) and other sphingolipids in breast cancer and normal breast tissue. The levels of the sphingolipids including sphingosine (Sph), dihydro-Sph (DHSph), S1P, dihydro-S1P (DHS1P) were determined by LC-ESI-MS/MS. Mean values are shown by horizontal lines.

Fig. 2.

Correlation between levels of sphingosine-1-phosphate (S1P) and other sphingolipids in breast cancer and those in normal breast tissue. The correlation between two variables are denoted by R^2 . $R^2 \geq 0.81$ is considered a large positive linear association and $R^2 \geq 0.20$ is considered a small positive linear association.

Fig. 3.

Levels of sphingomyelin (SM) in breast cancer and normal breast tissue. The levels of sphingomyelin (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, C26:0) were determined by LC-ESI-MS/MS. Mean values are shown by horizontal lines.

Fig. 4.

Correlation between levels of sphingomyelin in breast cancer and those in normal breast tissue. The correlation between two variables are denoted by R^2 . $R^2 \geq 0.81$ is considered a large positive linear association and $R^2 \geq 0.20$ is considered a small positive linear association.

Monohexosylceramides (HexCer)

Fig. 5.

Levels of monoheptosylceramides (HexCer) in breast cancer and normal breast tissue. The levels of HexCer (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, C26:0) were determined by LC-ESI-MS/MS. Mean values are shown by horizontal lines.

Fig. 6.

Correlation between levels of monohexosylceramides in breast cancer and those in normal breast tissue. The correlation between two variables are denoted by R^2 . $R^2 \geq 0.81$ is considered a large positive linear association and $R^2 \geq 0.20$ is considered a small positive linear association.

Fig. 7.

Levels of Ceramides in breast cancer and normal breast tissue. The levels of Ceramides (C14:0, C16:0, C18:1, C18:0, C20:0, C22:0, C24:1, C24:0, C26:1, C26:0) were determined by LC-ESI-MS/MS. Mean values are shown by horizontal lines.

Fig. 8.

Correlation between levels of ceramides in breast cancer and those in normal breast tissue. The correlation between two variables are denoted by R^2 . $R^2 \geq 0.81$ is considered a large positive linear association, and $R^2 \geq 0.20$ is considered a small positive linear association.