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# Clinical Relevance of Ceramide Metabolism in the Pathogenesis of Human Head and Neck Squamous Cell Carcinoma (HNSCC): Attenuation of $C_{18}$-ceramide in HNSCC Tumors Correlates with Lymphovascular Invasion and Nodal Metastasis

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## Abstract

It has been documented previously that defects in the generation of $C_{18}$-ceramide, a product of ceramide synthase 1 (CerS1), also known as longevity assurance gene 1 (hLASS1), play important roles in the pathogenesis and/or progression of HNSCC. However, whether altered levels of ceramide generation in HNSCC tumors have any clinical relevance remains unknown. In this study, the levels of endogenous ceramides were measured in tumor tissues of 45 HNSCC patients as compared to their normal tissues using high-pressure liquid chromatography/mass spectrometry (LC/MS), and then possible link between ceramide levels and the clinical parameters of HNSCC were examined. The data showed that the levels of $C_{16}$-, $C_{24}$-, $C_{24:1}$-ceramide were significantly elevated in the majority of tumor tissues compared to their normal tissues, while the levels of only $C_{18}$-ceramide were significantly decreased in HNSCC tumors, especially in tumor tissues of male patients. Importantly, it was also shown here that decreased $C_{18}$-ceramide levels in HNSCC tumor tissues were significantly associated with the higher incidences of lymphovascular invasion, and pathologic nodal metastasis. Importantly, attenuation of $C_{18}$-ceramide was also positively linked to the higher overall stages of the primary HNSCC tumors. Therefore, these data suggest, for the first time, that

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the defects in the generation/accumulation of $C_{18}$-ceramide might have important clinical roles in HNSCC, especially in lymphovascular invasion and nodal disease.

**Keywords**

Ceramide; Ceramide synthase; Longevity assurance gene (LASS); Head and neck cancer; Lymphovascular spread; Nodal metastasis

## 1. Introduction

Squamous cell carcinomas of the head and neck (HNSCC) are among the most aggressive group of cancers. Despite emerging new surgical techniques and chemoradiation protocols, HNSCC remains among the five leading causes of solid tumor-related deaths in the United States [1]. Five-year survival rates of patients with advanced stages of HNSCC remain around 50%, with little improvement for several decades. Although several tissue biomarkers such as p16, p53, cyclin D1, cyclo-oxygenase 2 (COX-2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and matrix metalloproteinases have been associated with HNSCC, there is still a need for new diagnostic and/or prognostic markers [2].

The biologically active sphingolipid ceramide has been a source of interest in the regulation of cancer cell growth and response to therapy. In addition to its important role as a key molecule in sphingolipid metabolism, ceramide also has important effector functions, which involve the regulation of apoptosis, cell cycle arrest, or senescence [3].

Previous studies have demonstrated a role for sphingolipids in the regulation of HNSCC growth and progression. For example, ceramide and sphingosine have been shown to inhibit EGF receptor kinase in epidermoid cell A431 carcinoma cells [4,5]. In addition, altering the levels of membrane glycolipids of human A431 cells by an inhibitor of glucosylceramide synthase resulted in a rapid loss of epithelial cell morphology, a reduced rate of cell growth, and inhibition of cell-substrate interaction [6]. In another study, treatment of squamous cell carcinoma cell line, DJM-1, with an exogenous ceramide promoted differentiation, and inhibited proliferation, suggesting a regulatory role of ceramide in growth and differentiation of keratinocytes [7]. Moreover, there have been additional studies which confirmed the active regulatory role of ceramide in HNSCC cells regarding the induction of apoptosis [8], EGF receptor modulation [9,10], reversing drug and radiation resistance [11,12], inhibition of neo-vascularization [13], and enhancing the anti-cancer actions of various chemotherapy agents [14,15], or photodynamic therapy [16].

Recently, treatment of human UM-SCC-22A (SCC of the hypopharynx) cells with a novel exogenous ceramide analogue, L-threo-C6-pyridinium-ceramide (L-t-C6-Pyr-Cer) resulted in a significant inhibition of telomerase activity, and prevented the growth of HNSCC xenografts in SCID mice in vivo [17]. It is well recognized that telomerase is found to be active in 80-90% of HNSCC and is proposed to be an essential step for cancer cell immortalization. Telomerase is also associated with poor prognosis of patients with HNSCC [18,19].

In another line of investigation, the functions of specific subspecies of endogenous ceramides with different fatty acid chain length in HNSCC growth and/or progression has been examined. Analysis of the levels of endogenous ceramides between tumor and normal mucosa tissues of the same patients with HNSCC showed that only $C_{18}$-ceramide levels were decreased in the majority of tumor tissues, whereas the levels of other ceramides, such as $C_{16}$-, and $C_{24}$-ceramides were increased in HNSCC tumor tissues when compared to their normal

counterparts [20]. Further experiments showed that overexpression of the mammalian homologue of the yeast longevity assurance gene 1 (LASS1), also referred to as ceramide synthase1 (CerS1), which is known to selectively generate $C_{18}$-ceramide, resulted in the inhibition of HNSCC cell growth, and enhanced chemotherapy-induced apoptosis in UMSCC22A cells *in situ*, and in HNSCC xenografts *in vivo* [20,21].

Although, these data demonstrated an important role for ceramide signaling in the regulation of HNSCC pathogenesis and/or progression both *in situ* and *in vivo*, the clinical relevance of endogenous ceramide levels in HNSCC is still unknown. Therefore, in this study, we examined the association between changes in the levels of endogenous ceramide and clinical parameters of HNSCC. The data demonstrated that alterations of the $C_{18}$-ceramide levels in HNSCC tumors are significantly correlated with the presence of lymphovascular invasion and nodal metastatic disease, suggesting that attenuation of $C_{18}$-ceramide is highly associated with the advanced HNSCC in the clinic.

## 2. Materials and methods

### 2.1. Clinical samples

With the permission of the Institutional Review Board, randomized tissue samples of 33 male (73%) and 12 female (27%) HNSCC patients were obtained from the tumor bank of the Hollings Cancer Center at Medical University of South Carolina. For each patient, paired tissue samples, obtained from the tumor or from the pathologically negative healthy mucosa near tumor site, were studied. Therefore, randomization was performed among the patients whose paired tissue specimens included both the pathologically documented tumor and healthy mucosa in the tumor bank. The demographics and clinicopathologic findings of the patients are shown in Table 1. The mean follow up time for the group was 15.1 months (ranging from 1 to 58 months).

### 2.2. Measurement of endogenous ceramides and sphingosine-1-phosphate (S1P)

Measurements of the ceramide subspecies and S1P in HNSCC tumor versus their normal tissues were performed by LC/MS as previously described [22]. The ceramide levels were normalized to total protein levels. The mRNA levels of human longevity assurance gene 1 and 6 (LASS1/CerS1 and LASS6/CerS6) in tumor and normal tissues of randomly selected 12 patients were measured using real-time PCR.

### 2.3. Analysis of the association between ceramide levels and clinical parameters

Electronic chart reviews of the patients were performed with data collection encompassing demographics, site and stage of the tumor, pathologic findings including nodal metastatic disease, perineuronal spread and lymphovascular invasion. The date of the surgery in which the tissues were sampled, is accepted as the reference day for data collection regardless of the previous or consecutive treatment or surgery results.

### 2.4. Statistical analysis

The statistical analysis was performed using SPSS 14.0 (SPSS, Chicago, IL) and SAS version 9.1 (SAS Inc., Cary, NC). Comparison of ceramide levels between tumor and normal tissues of the patients were analyzed using the paired T-Test. Correlation between age or stages of the tumors with ceramide levels were analyzed using standard and multiple linear regression methods. Comparison of the risk of nodal metastasis in patients with higher or lower tumor levels of $C_{18}$-ceramide as compared to their normal counterparts were performed using Fisher's exact-test. In these studies *P* value of .05 was considered significant.

## 3. Results

### 3.1. The levels of endogenous ceramides in HNSCC tumor tissues as compared to normal tissues

To examine the clinical relevance of ceramide, first the levels of endogenous ceramides were measured in 45 pairs of tissues (normal as compared to tumor tissues) obtained from patients with HNSCC using LC/MS, and results are presented in Table 2. Consistent with the previously published data [20], which were obtained from a smaller cohort (n=14 pairs), the current data in this study using a larger cohort of patients (n=45 pairs) showed that endogenous $C_{16}$-, $C_{24}$-, $C_{24:1}$-ceramides were significantly increased in tumor tissues whereas the levels of only $C_{18}$-ceramide (both $C_{18:0}$- and $C_{18:1}$-ceramides) were significantly decreased in HNSCC compared to their normal tissues (Table 2). The differences of other minor ceramides, such as $C_{14}$- and $C_{20}$-ceramides in tumor and normal tissue levels were not significant (Table 2). Thus, these data suggest that attenuation of $C_{18}$-ceramide, which is known to mediate anti-proliferative functions [20,21], in HNSCC tissues may play a role in the pathogenesis and/or progression of this disease.

Then, possible correlation between ceramide levels and clinical parameters, such as gender, lymphovascular invasion, nodal metastasis, and overall stage of the tumors were analyzed.

### 3.2. The analysis of relationship between ceramide levels and gender

The study group consisted of 12 female and 33 male patients. Interestingly, the levels of ceramide, in general, in the normal and tumor tissues of female patients were higher than that of males. Importantly, the data showed that $C_{18}$-ceramide was significantly lower in HNSCC tumor tissues, compared to their normal counterparts in the majority of the male patients ($P = .02$), whereas the levels of other ceramide species did not show any significant changes based on the gender of these patients (Table 3).

### 3.3. Decrease in $C_{18}$-ceramide in HNSCC tumor tissues significantly correlates with lymphovascular invasion and nodal metastasis

Among 45 patients with HNSCC included in this study, 14 patients were presented with lymphovascular invasion in their pathologic specimens, while 26 patients showed no lymphovascular invasion, and reports of 5 patients did not specify the presence or absence of this feature (Table 1). As shown in Table 4, the tumor levels of $C_{18}$-ceramide in patients who presented with lymphovascular invasion were significantly lower than the patients who did not ($P = .05$). Eight patients had higher $C_{18}$-ceramide levels in their tumor tissues compared to normal tissues, and impressively, none of these 8 patients were diagnosed with lymphovascular invasion. On the other hand, 14 patients of 32 (44%), whose tumor $C_{18}$-ceramide levels were lower than normal tissue levels presented with lymphovascular spread. The difference between two groups was also significant (Fisher's exact test, $P = .02$). The other ceramide subspecies in these tumors presented no significant changes regarding lymphovascular spread.

Retrospective reviews of the pathologic specimens revealed that 22 patients exhibited pathological presence of nodal metastasis at the time of surgery, and among these, 6 and 16 patients presented N1 and N2 neck disease, respectively (Table 1). Interestingly, all the ceramide levels were higher in tissue samples obtained from patients without nodal metastasis than in tissues of patients with nodal disease. More importantly, $C_{18}$-ceramide levels in tumors with nodal disease were significantly lower ($P = .02$) when compared to its levels in tumor tissues of patients without nodal metastasis (Table 5).

Importantly, regression analysis of the correlation between ceramide levels and the overall stages of the patients (presented in Table 1) showed that the difference in the levels of $C_{18}$-

ceramide between normal and tumor tissues (normal – tumor) is positively associated with higher overall stages of HNSCC ($P$ = .004), and these data are shown in Table 6.

Taken together, these data show for the first time that attenuation of $C_{18}$-ceramide in HNSCC tumors significantly associate with lymphovascular invasion and nodal metastasis, which are among the known parameters of advanced clinical disease. However, there were not enough clinical data to assess whether attenuation of $C_{18}$-ceramide is linked to overall survival and/or disease-free survival of these patients with HNSCC, which needs to be explored further.

### 3.4. The expression levels of human LASS1 and LASS6 in HNSCC

In order to obtain a mechanistic insight into the perturbations of ceramide species, the mRNA levels of human homologue of the yeast longevity assurance gene 1 (LASS1)/CerS1, which is known to be involved in the generation of $C_{18}$-ceramide, were also examined in 12 pairs of HNSCC tumor and normal tissues using real-time PCR. The results showed that decreased tumor levels of $C_{18}$-ceramide, which were detected in 9 of 12 patients, were also associated with decreased mRNA levels of LASS1 in 5 of these 9 patients. However, lower levels of $C_{18}$-ceramide in the remaining 4 patients did not correlate with LASS1/CerS1 mRNA levels (Figure 1). Interestingly, higher levels of $C_{16}$-ceramide in HNSCC tumors were highly linked to the increased mRNA levels of human LASS6/CerS6, which is implicated in the generation of $C_{16}$-ceramide. It was observed $C_{16}$-ceramide was increased in the HNSCC tumor tissues of 11 out of 12 patients, and induced LASS6/CerS6 mRNA levels correlated with increased $C_{16}$-ceramide levels in 8 of those 11 patients. However, there was no increase in LASS6 mRNA expression in 3 patients who had increased levels of $C_{16}$-ceramide in their tumor tissues (Figure 2). Therefore, these data demonstrate that in addition to transcriptional regulation of LASS1/CerS1 and LASS6/CerS6, there might be a post-transcriptional or -translational regulation of these proteins, or modulation of their (dihydro)ceramide synthase activities in some patients, which are involved in the down- or up-regulation of $C_{18}$- or $C_{16}$-ceramides, respectively.

## 4. Discussion

In this report, the clinical relevance of endogenous ceramides in 45 pairs of HNSCC tumor and non-cancerous (normal) tissues was examined. Consistent with the previous data [20], results presented here demonstrated that the levels of only $C_{18}$-ceramide was significantly lower in the tumor tissues of patients with HNSCC, whereas the levels of other major ceramides, especially $C_{16}$-, and $C_{24}$-ceramides, were higher in these tumors, when compared to their normal counterparts. Interestingly, levels of $C_{18}$-ceramide in HNSCC tumors appeared to be significantly altered in male patients, and, in general, female patients contained higher levels of ceramides in their tissues (both normal and tumor tissues) as compared to males. More impressively, the data revealed for the first time that the attenuation of $C_{18}$-ceramide in HNSCC tumor as compared to normal tissues significantly correlated with lymphovascular spread, and nodal metastatic disease, which are known to be independent markers of poor prognosis and advanced disease for HNSCC patients in the clinic.

Previously, an important study revealed that total ceramide levels, without any information about fatty acid chain length specificity, were inversely correlated with malignant progression of human astrocytomas, and associated with poor patient prognosis [23]. In an independent study, the data showed that defects in the generation of $C_{18}$-ceramide in the majority of HNSCC tumors might play a role in the pathogenesis of this disease, and that reconstitution of the levels of $C_{18}$-ceramide via hLASS1/CerS1 expression resulted in apoptosis, and enhanced chemotherapy-induced cell death in HNSCC both *in situ* and *in vivo* [20,21]. The data presented here also showed similar results, confirming that decreased levels of $C_{18}$-ceramide in HNSCC tumor tissues compared to their normal counterparts using larger cohort of patients might play important roles in the pathogenesis of HNSCC. More importantly, results presented here

showed also the clinical relevance of ceramide in HNSCC, revealing that the lower levels of $C_{18}$-ceramide in tumor tissues associate with lymphovascular invasion and nodal metastatic disease in patients with HNSCC, which links the altered $C_{18}$-ceramide generation and/or accumulation with the advanced clinical disease.

The different incidence rates of HNSCC, especially for larynx cancer, in men and women have been a subject of research, and it has been reported that there is a high ratio of male to female rates of the laryngeal cancer incidences, especially in the glottic tumors [24,25]. Even though there exists a controversy regarding the existence of sex hormone receptors in larynx [26-28], the voice changes after hormone replacement therapies support of the presence of a relationship between sex hormones and the larynx [29,30]. In parallel with these, our data showed a significant difference in ceramide levels between female and male patients. Specifically, ceramide levels in the tissues of female patients were higher than males, and more importantly, $C_{18}$-ceramide levels were significantly lower only in the HNSCC tumor tissues of the male patients, which is also consistent with the higher incidence rates of these cancers in males. The relationship between ceramide metabolism, HNSCC and gender has not been reported previously, and thus these data are novel and interesting. Although, the reason for increased levels of ceramide in females, and decreased $C_{18}$-ceramides in males are not clear, these findings of the presented study warrant further analysis.

Lymphovascular invasion of the tumor has been found to be positively associated with lymph node metastasis of HNSCC [31-34]. The negative effect of lymphovascular invasion on disease specific survival has been shown for HNSCC previously [35]. Moreover, existence of perivascular invasion reduces the time interval between surgery and development of recurrence [34]. Finally, recent studies suggest that in addition to advanced nodal stage and perineuronal invasion, lymphovascular spread is an independent prognostic variable for hypopharynx carcinomas, and a predictor of poor survival [36]. In the current study, tumors tissues of patients who were presented with lymphovascular invasion contained significantly lower levels of $C_{18}$-ceramide when compared to their normal tissues ($P = .05$). Remarkably, none of the eight (0/8) HNSCC patients whose tumor $C_{18}$-ceramide levels were higher than that of their normal tissues exhibited any detectable lymphovascular spread, again supporting the role of $C_{18}$-ceramide in its regulation.

Nodal metastatic disease is a well recognized independent factor for poor survival in different sites of HNSCC, including tongue, floor of mouth, oropharynx, hypopharynx and larynx [37-40]. The survival is hampered by the presence of extracapsular spread feature of the tumor [41,42]. Several clinicopathologic parameters have been associated with nodal disease, such as tumor depth [43], lymphovascular invasion [31-34], histological grade [44], tumor differentiation [45], tumor size [31], and double DNA aneuploidy [45]. In the presented study, patients who had a nodal disease exhibited significantly lower levels of $C_{18}$-ceramide in their tumors, compared to their normal tissues ($P = .02$). The patients whose tumor $C_{18}$-ceramide levels were higher than their normal tissues presented a lower risk of nodal disease (33.3%) as compared to patients whose tumor $C_{18}$-ceramide levels were lower than that of their normal tissues (56%).

However, mechanisms that regulate $C_{18}$-ceramide generation and/or accumulation in HNSCC, leading to its altered levels, are still unknown and need to be determined. The analysis of the mRNA levels of hLASS1/CerS1, which is known to generate mainly C18-ceramide [46,47], by real-time PCR showed that decreased tumor levels of $C_{18}$-ceramide, which were detected in 9 of 12 patients, were associated with decreased mRNA levels of LASS1/CerS1 in only about 50% of these patients (5 out of 9 patients). Therefore, these data demonstrate that in addition to transcriptional regulation of LASS1/CerS1, there might be a post-transcriptional and/or -translational regulation of LASS1/CerS1, or modulation of its (dihydro)ceramide

synthase activity in some HNSCC patients, which might be involved in the down- regulation of $C_{18}$-ceramide.

Similarly, the biochemical mechanisms involved in the ceramide-mediated regulation of lymphovascular invasion, and the consequences of altered levels of $C_{18}$-ceramide, leading to this feature in HNSCC, however, are still unknown, and will require further examination. Recent studies have elucidated a role for protein phosphatase 2A (PP2A) in the regulation of vascular invasion, and since ceramide is a known activator of PP2A [3], its attenuation in HNSCC tumors might lead to negative modulation of PP2A activity [13]. Although this might be a plausible possibility, whether $C_{18}$-ceramide specifically plays a role in the activation of PP2A, and whether further metabolism of $C_{18}$-ceramide into other complex lipids, might inhibit PP2A function, are still unknown. For example, it is possible that further metabolism of $C_{18}$-ceramide to generate pro-survival lipids [48], such as sphingosine-1-phosphate (S1P) and/or ceramide-1-phosphate (C1P), might be important in the increased incidences of lymphovascular invasion and nodal metastasis. It is well documented that increased generation of S1P by sphingosine kinase 1 (SK1) plays important roles in malignant transformation, alteration of ceramide-induced apoptosis, altered autophagy, and drug resistance [49-51]. However, our analysis of S1P levels in 18 pairs of HNSCC tumor tissues used in this study as compared to their normal counterparts did not reveal any statistically significant differences. The average values of S1P in HNSCC tumor and normal tissues were 3.50 and 3.23 pmol/mg of protein, respectively (n=18 pairs, and $P > .05$). Nevertheless, the levels of S1P in larger cohort of patients, especially in the serum of HNSCC patients as compared to that of healthy individuals should be determined to examine its possible clinical roles in the pathogenesis of HNSCC, since it might be readily secreted from squamous tissues into the blood, which is a known characteristic of S1P in some cell/tissue types [52]. Indeed, targeting of extracellular S1P has been shown to exert anti-proliferative functions against various cancers [53].

Similarly, it has been recently reported that generation of C1P by ceramide kinase (CK) plays a role in the induction of growth in various cell types [54,55]. Therefore, increased levels of C1P, especially C18-ceramide-1-phosphate, in HNSCC tissues and/or serum of patients with the disease might be very important to evaluate in future studies.

These data also demonstrated that the levels of $C_{16}$-ceramide were highly up-regulated in the majority of HNSCC tumor tissues. Although there were no significant correlation between increased $C_{16}$-ceramide and any of the clinical parameters tested, its modulation might still play very important roles in the pro-survival of HNSCC cells. Taken together, these data also suggest that endogenous ceramides with different fatty acid chain lengths, such as $C_{18}$- and $C_{16}$-ceramides, might play distinct roles in the regulation of cell growth and/or survival. Indeed, our recent data demonstrated that while the $C_{18}$-ceramide generated by hLASS1/CerS1 inhibits telomerase, which is one of the best studied negative prognostic markers of most cancers, including HNSCC, generation of $C_{16}$-ceramide via LASS6/CerS6 induced the activity of the promoter of the human telomerase reverse transcriptase (L.G. Wooten-Blanks, P. Song, C.E. Senkal, and B. Ogretmen, unpublished data). Nevertheless, specific roles of hLASS/CerS-generated $C_{18}$- and $C_{16}$-ceramides in the regulation of PP2A function, if any, are still unknown, and need to be determined. Therefore, differential sub-cellular localization/topology of these endogenous ceramides, and the regulation of their distinct down-stream targets might be important for their distinct biological functions in human cancers, which should be further defined.

In conclusion, this study demonstrates the clinical relevance of ceramide signaling in the pathogenesis of HNSCC, and reveal for the first time that lower levels of $C_{18}$-ceramide in HNSCC tumors as compared to normal tissues is significantly associated with lymphovascular invasion and nodal disease. Although there were not enough clinical data to evaluate the

possible link between $C_{18}$-ceramide levels and the overall survival, it is believed that this possibility should be determined in an independent trial with a prospective study design. Nevertheless, these data reveal that lower $C_{18}$-ceramide levels, which is known to be generated by the function of hLASS1/CerS1 [46,47], in HNSCC tumors is significantly correlated with the parameters of advanced clinical disease, and support the view that $C_{18}$-ceramide might play important roles in the regulation of HNSCC growth and/or pathogenesis in the clinic.

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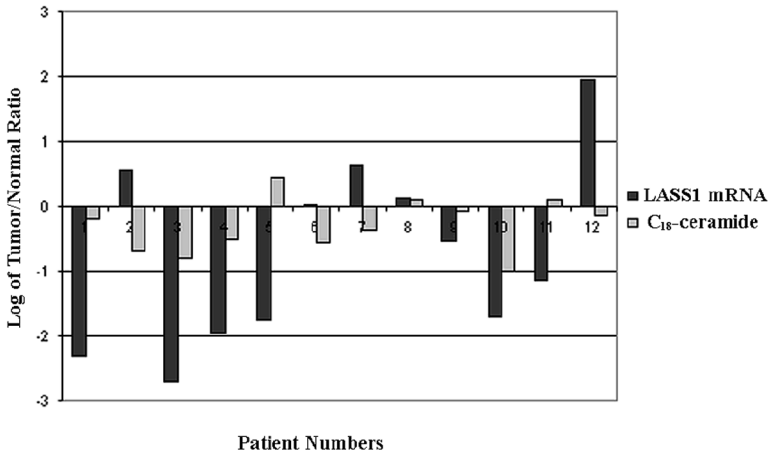


**Fig. 1.**

The alterations of LASS1/CerS1 mRNA and $C_{18}$-ceramide levels in HNSCC. The correlation between the expression levels of human LASS1/CerS1 mRNA, measured by real-time PCR, and $C_{18}$-ceramide levels, measured by LC/MS, in 12 HNSCC tumors as compared to their normal counterparts, was analyzed, and the fold-changes in their levels were presented in logarithmic scale. Negative numbers represent decreases in the levels of LASS1/CerS1 mRNA and ceramide levels. The measurements were performed in duplicates, and standard errors were within 0.5-1% of the presented values.

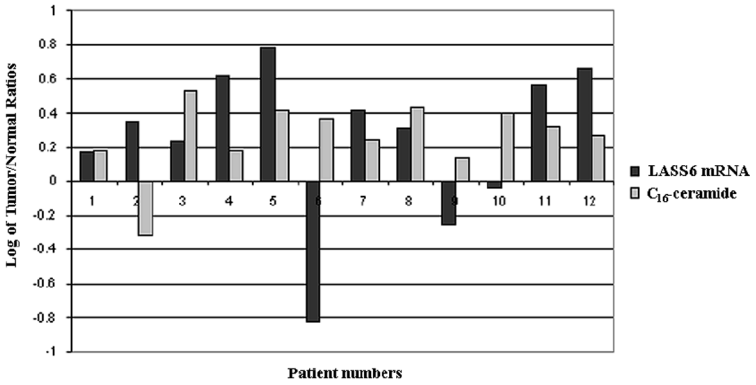


**Fig. 2.**

The alterations of LASS6/CerS6 mRNA and $C_{16}$-ceramide levels in HNSCC. The correlation between the expression levels of human LASS6/CerS6 mRNA, measured by real-time PCR, and $C_{16}$-ceramide levels, measured by LC/MS, in 12 HNSCC tumors as compared to their normal counterparts, was analyzed and presented in logarithmic scale. Negative numbers represent decreases in the levels of LASS6/CerS6 mRNA and ceramide levels. The measurements were performed in duplicates, and standard errors were within 0.5-1% of the presented values.

**Table 1**

Clinicopathologic parameters of 45 HNSCC patients

| Parameter | Number of patients | % of patients |
|---|---|---|
| Sex | | |
| Male | 33 | 73.3 |
| Female | 12 | 26.7 |
| Site | | |
| Oral cavity and Hypopharynx | 28 | 62.2 |
| Larynx | 10 | 22.2 |
| Sinonasal cavity | 5 | 11.1 |
| Unknown primary | 2 | 4.5 |
| T stage* | | |
| 1 | 3 | 6.7 |
| 2 | 7 | 15.5 |
| 3 | 6 | 13.3 |
| 4 | 13 | 28.9 |
| Recurrent | 14 | 31.1 |
| Unknown | 2 | 4.5 |
| N stage* | | |
| 0 | 21 | 46.6 |
| 1 | 6 | 13.3 |
| 2 | 16 | 35.6 |
| Undetermined | 2 | 4.5 |
| Overall stage* | | |
| 1,2 | 7 | 15.5 |
| 3,4 | 24 | 53.4 |
| Recurrent | 14 | 31.1 |
| Lymphovascular invasion | | |
| Absent | 26 | 57.8 |
| Present | 14 | 31.1 |
| Undetermined | 5 | 11.1 |
| Neural spread | | |
| Absent | 19 | 42.3 |
| Present | 20 | 44.4 |
| Undetermined | 6 | 13.3 |

*According to the guidelines of American Joint Committee of Cancer [21]

**Table 2**

Ceramide levels in tumor and counterpart normal tissues (pmol/1mg protein)

| **Ceramide subspecie** | **Normal Tissue level** * | **Tumor level*** | ***P*** ** |
|---|---|---|---|
| Total Ceramide | 275.43±229.94 | 391.61±311.06 | < .01 |
| $C_{14}$-ceramide | 13.12±22.0 | 20.05±18.21 | .07 |
| $C_{16}$-ceramide | 97.55±92.02 | 175.61±150.49 | <.01 |
| $C_{18}$-ceramide | 25.42±18.22 | 14.08±15.85 | <.01 |
| $C_{18:1}$-ceramide | 8.87±9.81 | 6.91±6.98 | .04 |
| $C_{20}$-ceramide | 10.59±7.81 | 9.86±7.83 | .63 |
| $C_{24}$-ceramide | 49.38±64.42 | 69.39±76.17 | <.01 |
| $C_{24:1}$-ceramide | 71.44±70.57 | 97.24±94.19 | .01 |

* Mean + SD

** Paired T test

**Table 3**

Ceramide levels* in female and male genders

| Subspecies | Female (n=12) | Male (n=33) | *P*** |
|---|---|---|---|
| Total Ceramide | | | |
| Normal | 374±299 | 240±192 | .08 |
| Tumor | 525±330 | 343±294 | .08 |
| $C_{16}$-ceramide | | | |
| Normal | 141±125 | 81.9±73.0 | .06 |
| Tumor | 231±177 | 155±137 | .14 |
| $C_{18}$-ceramide | | | |
| Normal | 31.2±25.1 | 23.3±15.0 | .21 |
| Tumor | 23.3±26.6 | 10.7±7.61 | .02 |
| $C_{18:1}$-ceramide | | | |
| Normal | 11.6±11.2 | 7.90±9.27 | .27 |
| Tumor | 10.2±8.47 | 5.71±6.08 | .06 |
| $C_{24}$-ceramide | | | |
| Normal | 50.3±50.6 | 47.7±68.9 | .91 |
| Tumor | 92.4±81.7 | 60.8±73.5 | .22 |
| $C_{24:1}$-ceramide | | | |
| Normal | 104±103 | 59.7±51.4 | .06 |
| Tumor | 130±98.4 | 85.5±91.3 | .17 |

* (pmol/1mg protein), mean+SD

** Unpaired T Test

**Table 4**

Ceramide levels * of patients with or without lymphovascular invasion

| Subspecies | Without Lymphovascular Invasion (n=26) | With Lymphovascular Invasion (n=14) | *P*** |
|---|---|---|---|
| Total Ceramide | | | |
| Normal | 258±193 | 328±317 | .39 |
| Tumor | 380±299 | 382±321 | .98 |
| $C_{16}$-ceramide | | | |
| Normal | 101±91.4 | 99.9±106 | .98 |
| Tumor | 183±140 | 144±112 | .38 |
| $C_{18}$-ceramide | | | |
| Normal | 25.0±18.1 | 26.5±19.7 | .81 |
| Tumor | 14.6±10.7 | 8.49±5.94 | .05 |
| $C_{18:1}$-ceramide | | | |
| Normal | 8.95±10.7 | 8.87±9.61 | .98 |
| Tumor | 7.33±7.59 | 6.13±6.02 | .61 |
| $C_{24}$-ceramide | | | |
| Normal | 42.3±67.3 | 60.7±64.9 | .41 |
| Tumor | 58.0±74.1 | 87.3±87.0 | .27 |
| $C_{24:1}$-ceramide | | | |
| Normal | 60.0±43.9 | 99.2±108 | .11 |
| Tumor | 87.2±90.3 | 112±111 | .45 |

* pmol/1mg protein, mean+SD

** Unpaired T Test

**Table 5**

Ceramide levels* of patients with or without nodal metastasis disease

| Subspecies | Without nodal metastasis (n=21) | With nodal metastasis (n=22) | *P*** |
|---|---|---|---|
| Total Ceramide | | | |
| Normal | 317±263 | 238±204 | .27 |
| Tumor | 392±265 | 362±325 | .74 |
| $C_{16}$-ceramide | | | |
| Normal | 114±110 | 77.8±72.5 | .21 |
| Tumor | 174±114 | 154±140 | .62 |
| $C_{18}$-ceramide | | | |
| Normal | 29.6±22.3 | 22.2±13.1 | .19 |
| Tumor | 15.8±11.2 | 9.12±6.25 | .02 |
| $C_{18:1}$-ceramide | | | |
| Normal | 11.7±12.1 | 6.55±6.94 | .09 |
| Tumor | 8.64±8.34 | 4.76±4.50 | .06 |
| $C_{24}$-ceramide | | | |
| Normal | 44.7±45 | 54.6±80.8 | .62 |
| Tumor | 69±70.6 | 71.7±85.4 | .91 |
| $C_{24:1}$-ceramide | | | |
| Normal | 87.7±87.5 | 57.9±51.5 | .18 |
| Tumor | 97.7±87 | 94.4±105 | .91 |

* pmol/1mg protein, mean+SD

** Unpaired T Test

**Table 6**

Regression model* for the $C_{18}$-ceramide difference between normal and tumor tissues

| Variable | Parameter Estimate | Standard Error | t-statistic | *P* |
|---|---|---|---|---|
| Intercept | -63.84101 | 24.49990 | -2.61 | 0.0150 |
| Age | 0.04165 | 0.32219 | 0.13 | .90 |
| Stage | 13.27076 | 4.27539 | 3.10 | < .01 |

* Only the effects for the variables alone were considered in this model; no interactions between the independent variables were considered.