Sphingosine Kinase 1 (SphK1) – A Novel Marker for Cancer Risk Assessment in Oral Premalignancy

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ABSTRACT

Introduction: Head and neck cancer is the sixth most common cancer world-wide, resulting in approximately 550,000 diagnoses of new cases and 300,000 deaths per year globally. Overexpression of Sphingosine kinase - 1 (SphK1) is found in head and neck squamous cell carcinoma (HNSCC) from early to advanced stages and is associated with tumor progression, invasion, metastasis and poor prognosis. This study assesses the expression of sphingosine kinase - 1 (SphK1) in oral leukoplakia and oral squamous cell carcinoma (OSCC) and suggests its role as a potential biomarker tool for cancer risk assessment in oral leukoplakia.

Methods: In this retrospective study, eighty-two (n = 82) archival formalin-fixed paraffin blocks consisting of 10 normal tongue mucosa (Group A), 42 cases of oral leukoplakia (Group B) and 30 cases of OSCCs (Group C) were selected and subjected to immunohistochemical staining for anti-rabbit SphK1 antibody. The three different groups were compared for the presence of Sphk1 expression. The clinicopathological parameters such as age, gender, habits, histopathology, and Sphk1 expression were analyzed and compared between the malignantly transformed leukoplakia with untransformed leukoplakia.

Results: Positive SphK1 expression was found in 18 out of 42 (42.9 %) cases of oral leukoplakia and 17 out of 30 (56.7 %) cases of OSCCs while there was no SphK1 expression in normal tongue mucosa. The expression of SphK1 among three different groups of tissue samples was statistically significant (P = 0.007). The correlation between the malignant transformed and untransformed leukoplakia lesion with respect to SphK1 expression was also found to be statistically significant (P = 0.007)

Conclusions: Positive SphK1 expression in oral leukoplakia is suggestive of an increased risk for malignant transformation which can be used as a biomarker tool. Higher SPHK1 expression in the OSCC may suggest an important role in the early stages of tumorigenesis.

INTRODUCTION

Most of the head and neck cancer are of squamous cell origin, of which, oral squamous cell carcinoma(OSCC) is the most common malignant tumor of the head and neck region. 1, 2 Generally OSCC is preceded by potentially malignant disorders (premalignant lesions) such as leukoplakia, erythroplakia. “Leukoplakia is the white
plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer and is the most common premalignant lesion found in mouth. The malignant transformation rate of oral leukoplakia (OLP) has been found between 0.13-34%. The gold standard method of assessing the malignant transformation risk of leukoplakia is the histopathological assessment of dysplasia. However, it has been found that most of the oral cancer developed from lesions that lacked dysplastic changes. Therefore there is a need to identify the molecular markers that can help in defining the risk potential of the precancerous lesions such as leukoplakia.

Sphingolipids are lipids present in cell membrane providing structural support and having key roles as signaling molecules. Ceramide and Sphingosine 1-Phosphate (S1P), have been found to have a critical role in regulation of cell proliferation, differentiation, angiogenesis, and survival in opposite directions. S1P regulates growth and survival (anti-apoptosis) while ceramide and sphingosine mediates growth arrest and cell death (apoptosis). Since these metabolites are interconvertible, their relative levels rather than absolute amount decides cell life. The “Sphingolipid rheostat” key modulators are Sphingosine kinases (SphKs) and Sphingosine 1-phosphate phosphatases (SPPs) which have exactly opposite action. Thus, the relative level of ceramide and S1P is important and balance of each other in cells is important determinant for the cell fate in either direction of cell growth inhibition or survival. The enzyme, sphingosine kinase (SphK), plays an important role in balancing this critical situation, which converts sphingosine to S1P by phosphorylating sphingosine. Two isoforms of sphingosine kinases, SphK1 and SphK2, are till date defined in human cells. SphK2 role in cancer development and pathogenesis still lack sufficient studies while SphK1 has been widely studied in different solid tumors, lung, breast, ovary, prostrate, stomach, uterus.

SphK1 is activated by variety of stimuli. They may be different growth factor receptors tyrosine kinases such as epidermal growth factor (EGF), we investigated the role of sphingosine kinase type 1 (SphK1 platelet derived growth factor), vascular endothelial growth factor (VEGF), evidence is presented for VEGF stimulation of sphingosine kinase (SPK tumor necrosis factor a (TNF-a), including activation of NF-kappa B, JNK, and antiapoptosis. We investigated how TRAF2 mediates differentially the distinct downstream signals. We now report a novel mechanism of TRAF2-mediated signal transduction revealed by an association of TRAF2 with sphingosine kinase (SphK and interleukin-1 (IL-1), and cross linking of the high affinity IgE receptors, which leads to generation and secretion of the potent sphingolipid mediator, sphingosine-1-phosphate (S1P vitamin D3, TGF-b, and ligands for G-protein coupled receptor such as acetylcholine, prosaposin, increased DNA synthesis, and prevented cell apoptosis. Prosaposin treatment induced pheochromocytoma cells (PC12 S1P itself activates SphK1 through a specific GPCR. Activation of SphK1 by diverse agonists and stimuli are well studied.

SphK1 was slightly highly expressed in normal mucosa adjacent to SCC than normal mucosa. SphK1 staining was found in all stages of HNSCC, even in stage I, and there was no differences between SphK1 staining from stage I to IV, which suggested the authors to conclude that SphK1 is most likely involved in early stages of malignant transformation from normal mucosa to HNSCC. In another study too, it was shown that SphK1 was found to be overexpressed in malignant tissue compared with nonmalignant tissue. Thus, the premalignant tissue expression of SphK1 may suggest an early malignant transformation potential of the lesion and must not be overlooked.

The purpose of this study was to determine the SphK1 expression pattern in premalignant lesion and OSCC. The knowledge of expression of SphK1 in premalignant lesion and the risk of malignant transformation of leukoplakia may be beneficial diagnostically as well as prognostically in oral cancer management.

METHODS

In this retrospective study, 82 formalin fixed paraffin embedded blocks were retrieved after scanning the archives in the Department of Oral Pathology, Tianjin Stomatology Hospital of Nankai University, Tianjin, China, of histopathologically reported cases of 10 normal mucosa of tongue, 42 OLP and 30 cases of OSCC and were divided into Groups A, B, and C respectively.
All 82 tissue specimens were considered for immunohistochemical staining. The Primary antibody Anti-rabbit SphK1 antibody (ABCAM-US) and secondary antibody Anti-rabbit/horse radish peroxide (HRP)/conjugated antibody IHC detection kit (ZSGB-Bio, Zhongshan-GBI Biotechnology Company Ltd., Beijing, China), was used. The ethical committee of the hospital approved the study.

In brief, the formalin-fixed, paraffin-embedded tissues (FFPE) were cut into 5 μm thick sections and were deparaffinized by fresh xylene, followed by dehydration in graded alcohol. Antigen retrieval was done by heating 10 min in microwave oven with citrate buffer (pH 8.0). The tissue sections were incubated overnight at 4 °C in refrigerator with Anti-rabbit SphK1 primary antibody at 1: 100 dilution. After 10 min wash in Phosphate Buffer Saline (PBS, pH 7.3), all the sections were incubated with secondary antibody, Anti-rabbit Horse radish peroxidase (HRP) conjugated antibody at 37 °C, for 30 minutes. Then the sections were incubated with diaminobenzidine (DAB) chromogen as substrate for 5-10 min. Finally; slides were washed and counterstained with hematoxylin and mounted with cover slip using a standard medium.

The criteria for inclusion into malignant leukoplakia was white lesions that were confirmed by histopathology in first visit and upon subsequent follow up, the lesion developed into OSCC confirmed by the histopathology at the same site. An untransformed leukoplakia inclusion criterion were white lesions confirmed as leukoplakia by histopathology. In the follow up period, patient did not develop cancer in the same lesion at the same site.

SphK1 expression was observed in the cytoplasm and plasma membrane of the cells. SphK1 was mainly localized in cytoplasm and cell membrane. The proportion of positively stained cells and intensity of staining was calculated. The percentage of positively stained cells were scored as score 0= no staining cells, score 1 = < 25 % positive staining cells, score 2 = 26 %~50 % positive staining cells, score 3 = 51 %~74 % positive staining cells, score 4= >74 % positive staining cells.

Staining intensity was scored as score 0 - no staining, score 1 - weak staining, score 2 - moderate staining, score 3 - strong staining. The staining index was the product of positively stained cells score and the staining intensity score. Under the light microscope, two oral pathologists evaluated and analyzed the slides.

All statistical analyses were carried out using the SPSS 21.0 statistical software package. All the parameters were tabulated and the expression of SphK1 was analyzed statistically among different groups using Pearson Chi-square test. The clinicopathological parameters details such as age, sex, habits, histopathology and SphK1 expression between malignant transformed leukoplakia and untransformed leukoplakia were analyzed and compared were. The screening test for the SphK1 expression was used. The significance level was set at $P <0.05$.

**RESULTS**

The study results showed, absence of SphK1 expression in the epithelium of all the normal tongue samples (Group A). Positive SphK1 expression was noticed in 18 (42.9 %) out of 42 cases of leukoplakia (Group B) and 17 (56.7 %) out of 30 cases of OSCCs (Group C). The expression of SphK1 among different groups was significant ($P = 0.007$) as shown in table 1. The correlation between the malignant transformed and untransformed leukoplakia lesion with respect to SphK1 expression was statistically significant ($P = 0.007$) but it was not statically significant with respect to other clinicopathological parameters such as age, sex, smoking, and alcohol intake and histopathology status ($P> 0.05$).

The patient characteristics of 42 leukoplakia cases were studied. Twenty-six patients (62 %) were women and the remaining 16 (38 %) were men. With regard to age, 24 (57.1 %) were above or equal to 60 years while 18 (42.9 %) were below age 60. Twenty-six of 42 patients (62 %) were smokers and 23 (54.8 %) were habitual alcohol drinkers. The mean tobacco consumption was 20 cigarettes a day. Twenty-seven of 42 patients (64.2 %) were found to be having dysplasia and the remaining 15 (35.8 %) as having only hyperplasia. 18 (42.9 %) leukoplakia cases were found to be SphK1 positive. The baseline characteristics of study participants are presented in Table 2.
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### Table 1. Expression of SphK1.

| Groups          | Number | SphK1 Expression | X²      | P value |
|-----------------|--------|------------------|---------|---------|
|                 |        | Positive (%)     | Negative (%) |
| A. Normal tissue| 10     | 0 (0%)           | 10 (100%) | 9.845   | 0.007   |
| B. Leukoplakia  | 42     | 18 (42.9%)       | 24 (57.1%)|
| C. OSCC         | 30     | 17 (56.7%)       | 13 (43.3%)|

### Table 2. Association between patient’s baseline characters and Leukoplakia status.

|                          | Untransformed Leukoplakia (N=24) | Malignant transformed leukoplakia (N =18) | P value |
|--------------------------|----------------------------------|------------------------------------------|---------|
| Age, number (%)          |                                  |                                          |         |
| ≥60 years                | 14 (58.3)                        | 10 (55.6)                                | 0.857   |
| <60 years                | 10 (41.7)                        | 8 (44.4)                                 |         |
| Gender, number (%)       |                                  |                                          |         |
| Male                     | 8 (33.3)                         | 8 (44.4)                                 | 0.463   |
| Female                   | 16 (66.7)                        | 10 (55.6)                                |         |
| Smoking, number (%)      |                                  |                                          |         |
| Yes                      | 15 (62.5)                        | 11 (61.1)                                | 0.927   |
| No                       | 9 (37.5)                         | 7 (38.9)                                 |         |
| Alcohol Intake, number (%)|                                 |                                          |         |
| Yes                      | 12 (50)                          | 11 (61.1)                                | 0.474   |
| No                       | 12 (50)                          | 7 (38.9)                                 |         |
| Histopathology, number (%)|                                 |                                          |         |
| Hyperplasia              | 10 (41.7)                        | 5 (27.8)                                 | 0.353   |
| Dysplasia                | 14 (58.3)                        | 13 (72.2)                                |         |
| SphK1 Expression, number (%)|                               |                                          |         |
| Positive                 | 6 (25)                           | 12 (66.7)                                | 0.007   |
| Negative                 | 18 (75)                          | 6 (33.3)                                 |         |

Immunohistochemical analysis of SphK1 expression was performed on all 82-tissue section. SphK1 protein was detected mostly in the cytoplasm and to some extent in membranes in leukoplakia and OSCC. In contrast, SphK1 expression in normal tongue epithelium was negligible or absent (Fig. 1A). SphK1 expression in oral leukoplakia tissue was highly variable; some of the lesion had no expression while some had abundantly expressed (Fig. 1B-C). Out of 30 OSCC lesions, 17 (56.7 %) lesions showed highly positively SphK1 expression (Fig. 1D). The positive expression rate of SphK1 protein in oral squamous cell carcinoma of tongue (56.7 %) was significantly higher than that in leukoplakia tissues (42.9 %) and normal tongue epithelium (0 %), and the expression of SphK1 protein in leukoplakia tissues was significantly higher than that in normal tongue tissue. There was statistical significant difference in SphK1 expression between the three groups ($X^2= 9.845, P =0.007$) (Table 1).

A correlation between untransformed leukoplakia and malignant transformed with respect to a number of clinicopathological parameters were analyzed (Table 2). There was no statistically significant correlation with regards to age of the patients ($P = 0.857$), gender ($P = 0.463$), lifestyle habits such as smoking ($P = 0.927$), ethanol intake ($P = 0.474$) and histopathology ($P =0.353$). The expression of SphK1 was correlated...
with untransformed leukoplakia and malignant transformed leukoplakia (Table 2). Out of 42-leukoplakia lesion, the Sphk1 expression was found positive in 18 (42.9 %) lesions while 24 (57.1 %) lesions were negative. Of the 18 malignant transformed leukoplakia, 12 (66.7 %) cases were SphK1 positive whereas only 6 (33.3 %) were SphK1 negative. Likewise, out of 18 SphK1 positive leukoplakia cases, 12 transformed into malignant lesions whereas, out of 24 SphK1 negative leukoplakia only 6 cases transformed into malignant lesion. There was statistically significant correlation between the transformed and untransformed leukoplakia lesion with respect to SphK1 expression (P = 0.007).

**DISCUSSION**

Ninety percent head and neck cancer generally begins from the squamous cells lining the mucosal surface present inside the head and neck, thus referred as Head and Neck squamous cell carcinoma (HNSCC).1,2 The specific oral cavity ones is called as Oral Squamous cell carcinoma (OSCC). HNSCC is the sixth most common cancer worldwide, resulting in approximately 550,000 diagnoses of new cases and 300,000 deaths per year 29 with recurrence rates of advanced stage cancer greater than 50%.30 In spite of great stride in cancer therapy, the high rate of recurrence resulting in high morbidity and mortality of advanced cases is a challenging effort. Thus, new therapeutic measures are needed for cancer management in molecular diagnostic area identifying new targets that have significant role in cancer growth and metastasis. Expression of SphK1 in premalignant lesion and OSCC may offer insight into possible use of this enzyme as a biomarker for diagnosis and prognosis of HNSCC.

The oncogenic role of SphK1 was first noted by Xia and colleagues in 2000 where high expression of SphK1 in NIH3T3 fibroblast cells promoted cell proliferation and tumor formation in NOD/SCID mice.31 The overexpression or up-regulation of SphK1 has shown cell proliferation, cell survival, anti-apoptotic action and promotion of tumor progression in mice.32-34 It has been evident that SphK1 down-regulation in glioblastoma cells and breast cancer cells arrest cell cycle and inhibiting SphK1 using a dominant-negative form of SphK1 inhibited tumor formation in nude mice.16, 34-36 sphingosine kinase (SphK) The high expression of SphK1 was correlated with poor survival outcomes in head and neck tumors 37-38 and other tumors of the body like glioblastoma 35 and breast cancer.39

It has been documented that patients with high SphK1 expression had marked shorter survival rate compared with those with low expression of SphK1 at either early stages or advanced stages, suggesting that SphK1 is a valuable prognostic marker for NPC (nasopharyngeal carcinoma) patients at all diseases stages.37 In another study, human esophageal carcinoma tissue samples of 124 patients diagnosed of esophageal squamous cell carcinoma (ESCC) were stained for SPHK1 and scored by intensity
and percentage of staining on a scale of 1 to 3, which corresponded to < 25 %, 25 % - 50 %, >50 % staining, respectively. Kaplan Meier analysis revealed that SphK1 expression (score ≥ 2) was significantly associated with clinical failure (three year survival), while weak SphK1 staining (score <2) was associated with longer survival (eight years). SphK1 was over-expressed in tumor tissues compared with normal tissue when measured with Western Blot and IHC. It was also note that SphK1 expression was significantly related with depth of tumor invasion, lymph node metastasis, and pathological state.

The mechanism of action of SphK1 in carcinogenesis has been a topic of debate. Meller et al. in 2008 suggested that TGF-beta activates SphK1 leading to increase intracellular S1P, which in turn activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) inducing migration and invasion of cells. Similarly cytoprotective effect of S1P on HNSCC epithelial cells also as well as in keratinocytes also suggest poor prognosis of SphK1 expressed cells. Furthermore, numerous study has shown that Ceramide induces apoptosis. However increase SphK1 leads to decrease ceramide level halting apoptosis mechanism and favoring S1P induced cell growth and survival (anti-apoptosis) which supports with the association of SphK1 expression with poor prognosis in HNSCC. Similarly, C18-ceramide in HNSCC tumors was lower compared to normal tissues and was correlated with lymphovascular invasion and nodal metastases. The decrease in ceramide level may be due to increase SphK1 level leading to tumor progression and poor prognosis.

Different markers of dysplastic leukoplakia have been associated with malignant transformation. Different studies have elicited the association of podoplanin with malignant transformation of oral leukoplakia. De Vicente et al. illustrated significant association of podoplanin expression to the grade of dysplasia (P<0.0005), and with the risk of progression from dysplasia to oral cancer (P< 0.0005). Podoplanin expression, epithelial dysplasia and smoking habit had association to malignant transformation of oral leukoplakia in univariate analysis. However, in multivariate Cox regression model, it was found that only podoplanin expression was independent predictor of oral cancer development (HR = 8.738; 95% CI = 1.83–41.63; P = 0.007).

In another study, Von Zeidler et al. studied E-cadherin, a cell membrane protein involved in cell-cell adhesion, expression in normal mucosa, oral leukoplakia and OSCC and noted significant decrease in E-cadherin expression even in early stage of dysplasia. Loss of epithelial cohesion may be the risk of malignant transformation of leukoplakia. TGF-a and EGFR overexpression in leukoplakia with dysplasia has also been demonstrated as early marker of malignancy.

Three parameters such as oral pre-malignant lesion histology, cancer history, biomarkers such as chromosomal polysomy, p53 protein expression, and loss of heterozygosity at chromosome 3p or 9p were the best indicator of risk of malignant transformation of precancerous lesion. It has been reported that the risk factors of malignant transformation of oral leukoplakia include the advanced age, grade of dysplasia, female sex, leukoplakia exceeding 200 mm² and non-homogeneous type (e.g. erythroplakia).

The gold standard method for judging whether the leukoplakia has turned into malignant or not is by the study of dysplastic changes. However, studies have highlighted the significant subjective inter-observer and intra-observer variation in reporting epithelial dysplasia. Moreover, 14 out of 27 dysplastic leukoplakia samples in our study patients didn’t develop OSCC. Therefore, immunohistochemical staining of SphK1 along with histopathological assessment would be better for evaluating malignant transformation risk in OLP compared with only histopathological assessment of epithelial dysplasia.

CONCLUSIONS

In conclusion, cancer of oral cavity results in debilitating morbid condition, poor quality of life, functional and esthetical compromise and poor prognosis. Majority of oral cancer develop from precancerous lesion or potentially malignant disorders. The in-depth understating of these premalignant lesions and the risk of malignant transformation must be accurately measured.
The SphK1 expression in premalignant lesion may be an early sign of malignant transformation that must not be overlooked. Along with histopathological assessment of dysplastic lesion, SphK1 can be used as a potential biomarker for evaluating the risk of malignant transformation in leukoplakia. However, further large-scale studies might be sought upon to fully elucidate the role of SphK1 in malignant transformation and oral cancer initiation and progression. Our study indicates that SphK1 expression in oral epithelium is an early event in oral tumorigenesis and predicts the evolution of pre-neoplastic lesions and malignant transformation risk potential.

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