Biochemical and Biophysical Research Communications 460 (2015) 341–347

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

High expression of sphingosine kinase 1 is associated with poor prognosis in nasopharyngeal carcinoma

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ARTICLE INFO

Article history:
Received 4 March 2015
Available online 14 March 2015

Keywords:
SPHK1
Nasopharyngeal carcinoma
Prognosis

ABSTRACT

It has been reported that sphingosine kinase 1 (SPHK1), an oncogenic enzyme, was involved in the development and progression of a number of human cancers. However, the role of SPHK1 in nasopharyngeal carcinoma (NPC) is largely unknown. The present study aimed to characterize the expression of SPHK1 in human NPC and evaluate its clinical significance. Real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and Western blot analyses showed that the expression of SPHK1 mRNA and protein in NPC specimens was significantly higher than that in non-tumorous nasopharyngeal mucosa biopsies. Immunohistochemistry (IHC) was conducted to characterize the expression pattern of SPHK1 in 142 archived paraffin-embedded NPC specimens. Statistical analyses revealed that high levels of SPHK1 expression were associated with the clinical stages, locoregional recurrence and distant metastasis of NPC. NPC patients with high levels of SPHK1 expression had shorter survival time, whereas those with lower levels of SPHK1 expression survived longer. Moreover, multivariate analysis suggested that SPHK1 up-regulation was an independent prognostic factor for NPC. Our results suggest for the first time that SPHK1 is involved in the development and progression of NPC, which can be used as a useful prognostic marker for NPC patients and may be an effective target for treating NPC.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China and Southeast Asia [1,2]. Due to its highly invasive and metastatic features, NPC receive more concerns than other head and neck malignancies [3]. While NPC is sensitive to radiotherapy, the 5-year survival rate of NPC patients remains between 50 and 60%. Distant recurrence is the major reason causing treatment failures [4]. Currently, the evaluation of NPC prognosis is primarily based on the TNM stage, however, NPC patients of the same clinical stage often have different clinical outcomes. Therefore, it has been suggested that TNM staging is inadequate or inappropriate for predicting the prognosis of NPC [5,6]. The molecular mechanisms underlying the development and progression of NPC remain largely unknown. Therefore, investigation of the pathogenesis and identification of molecular markers of NPC may facilitate early diagnosis, prognosis prediction, and development of effective therapeutic strategies for NPC patients.

Sphingosine kinase (SPHK), the rate-limiting enzyme of sphingosine 1 phosphate (S1P) synthesis, closely regulates the ceramide/sphingosine-S1P rheostat [7]. Two functional SPHK isoenzymes, SPHK1 and SPHK2, have been identified in humans thus far [8–10]. SPHKs can be activated by tumor necrosis factor-α (TNF-α), tyrosine kinase growth factors, and G-proteincoupled receptor ligands [11–14] and are involved in a number of fundamentally biological processes such as cell proliferation, antiapoptosis, angiogenesis, inflammation, and cell invasion [15–20]. It has been shown that activation of SPHK1 contributed to tumorigenesis by improving the proliferation, antiapoptosis, and transformation of tumor cells [21–23]. The oncogenic role of SPHK1 was first reported in a study conducted by Xia and colleagues in 2000, in which SphK1

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http://dx.doi.org/10.1016/j.bbrc.2015.03.036
0006-291X/

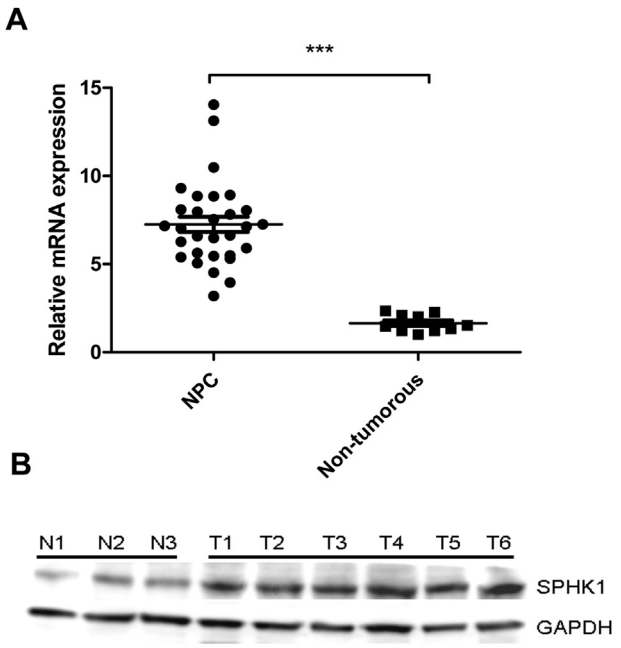

Fig. 1. SPHK1 overexpression in NPC specimens detected by quantitative RT-PCR and Western blotting analyses. (A) The mRNA levels of SPHK1 in 30 NPC biopsies and 10 non-tumorous nasopharyngeal epithelial tissues were determined by qRT-PCR. β-actin was used as an internal control. (B) The SPHK1 protein levels in 3 non-tumorous nasopharyngeal epithelial tissues (N1–N3) and 6 NPC biopsies (T1-T6) were evaluated by Western blotting assay. GAPDH was used as a loading control. (***, $P < 0.001$).

overexpression in NIH3T3 cells resulted in transformed phenotypes and promoted tumor formation in NOD/SCID mice [24]. In addition, a dominant-negative form of SPHK1 inhibited cell proliferation and suppressed tumor formation in nude mice [21,25,26]. Furthermore, previous studies have identified SPHK1 overexpression in a variety of human solid tumors [27–32]. However, the expression patterns and clinical significance of SPHK1 in NPC has not been addressed. The aim of the present study was to investigate the clinical significance of SPHK1 expression in NPC.

2. Materials and methods

2.1. Tissue specimens

Fresh tumor specimens used for qRT-PCR and Western blotting analyses were collected from NPC patients who had undergone biopsies at the Southwest Hospital of Third Military Medical University (Chongqing, China) during 2014. For IHC analysis, a total of 142 archived paraffin-embedded NPC samples and 10 non-tumorous nasopharyngeal tissues were collected between January 2007 and August 2009. Clinical stages and histological subtypes were determined by pathologists in the department of pathology at the Southwest Hospital according to the 6th edition of the TNM Classification of the UICC (International Union Against Cancer). All the patients were followed from the date of diagnosis to death or the lasted census date. This study was approved and supervised by the ethical committee of the Southwest Hospital.

2.2. Real-time quantitative reverse transcriptase–polymerase chain reaction

The total RNA was extracted using the Trizol Reagent (TaKaRa, Japan) according to the manufacturer's protocol. RNAse-free DNase I was used to eliminate DNA contamination. The isolated RNA was quantified using a NanoDrop spectrophotometer (Agilent Technologies, USA). Complementary DNA (cDNA) reversely transcribed from 500 ng RNA by a Reverse Transcription Kit (TaKaRa, Japan) was used as templates for polymerase chain reaction (PCR) amplification (40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s) using a SYBR Premix ExTaqII kit (TaKaRa, Japan) according to the manufacturer's instruction. β-actin was used as an internal control. Sequences of the real-time PCR primers are as follows: SPHK1 forward primer 5′-CTTGCAGCTCTTCCGGAGTC-3′, SPHK1 reverse primer 5′-GCTCAGTGAGCATCAGCGTG-3′, β-actin forward primer 5′-GACAGGATGCAGAAGGAGATTACT-3′, β-actin reverse primer 5′-TGATCCACATCTGCTGGAAGGT-3′. The $2^{-\Delta\Delta Ct}$ method was used to calculate expression relative to the β-actin housekeeping control [33].

2.3. Western blotting

The total protein was extracted from NPC fresh tissues using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford) and quantified using a BCATM Protein Assay (Pierce). The proteins (50ug) were separated in sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred onto polyvinylidene fluoride membranes (Milipore) for immunoblotting. The membrane was incubated with anti-SPHK1 rabbit antibody (1:500; Abcam). Anti-GAPDH antibody (1:1000 dilution; Abcam) was used as the loading control. The results were visualized using a chemiluminescent detection system (Pierce ECL Substrate Western Blot Detection System; Thermo Scientific, Rockford, Ill) and exposure to autoradiography film (Kodak XAR film).

2.4. Immunohistochemical staining

Immunohistochemistry was performed as previously described [34]. SPHK1 antibody (1:100 dilution, Abcam) was used as the primary antibody. Immunohistochemistry (IHC) results were independently examined and scored by two pathologists, who were blinded to the histopathologic features and clinical data of the patients. The IHC scores were determined on the basis of the proportion of positively stained tumor cells and the staining intensity. The proportion of positively stained tumor cells was graded from 0 to 4 (0, <5% positive cells; 1, 5–25 %; 2, 26–50 %; 3, 51–75 %; 4, >75%). The staining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The staining index (0–12) generated by multiplying the scores of the proportion of positively stained tumor cells and staining intensity was used to evaluate the expression of SPHK1 in tumor and non-tumorous tissues. The high and low expression level was defined based on a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival. Specifically, the high or low expression of SPHK1 was identified as a staining index score ≥ 6 and ≤ 4, respectively.

2.5. Statistical analysis

All data were analyzed using the SPSS statistical software (version 17.0, SPSS Chicago, USA). The association between SPHK1 expression and clinicopathological features of NPC patients was assessed by chi-square test. The survival curves of NPC patients with high or low SPHK1 expression were plotted using the Kaplan–Meier analysis and log-rank test. Univariate and multivariate regression analysis were performed with the Cox proportional hazards regression model to determine the effects of potential prognostic factors on survival. $P < 0.05$ was considered as statistically significant.