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

Wenhua Li [a], Zhiqiang Tian [b], Hong Qin [a], Ni Li [a], Xiaoqing Zhou [c], Jian Li [d, *], Bing Ni [b, *], Zhihua Ruan [a, *]

[a] Department of Oncology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China
[b] Institute of Immunology, PLA, Third Military Medical University, Chongqing 400038, PR China
[c] Department of Otolaryngology, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China
[d] Institute of Hepatopancreatobiliary Surgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China

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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

* Corresponding authors. Fax: +86 23 68772348.
E-mail addresses: lijian_165@126.com (J. Li), nibingxi@yahoo.com (B. Ni), rzh1234@163.com (Z. Ruan).

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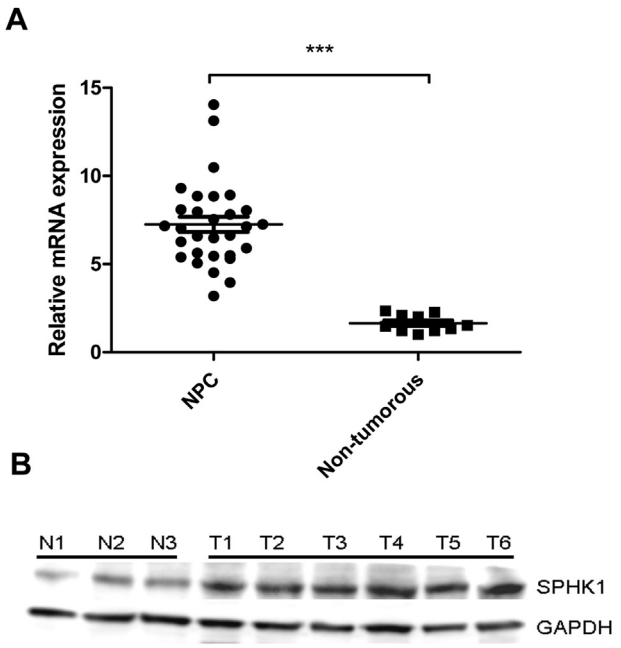

Fig. 1. SPHK1 overexpression in NPC specimens detected by quantitative RT-PCR and Western blotting analyses. (A) The mRNA levels of SPHK1 in 30 NPC biopsies and 10 non-tumorous nasopharyngeal epithelial tissues were determined by qRT-PCR. β-actin was used as an internal control. (B) The SPHK1 protein 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.

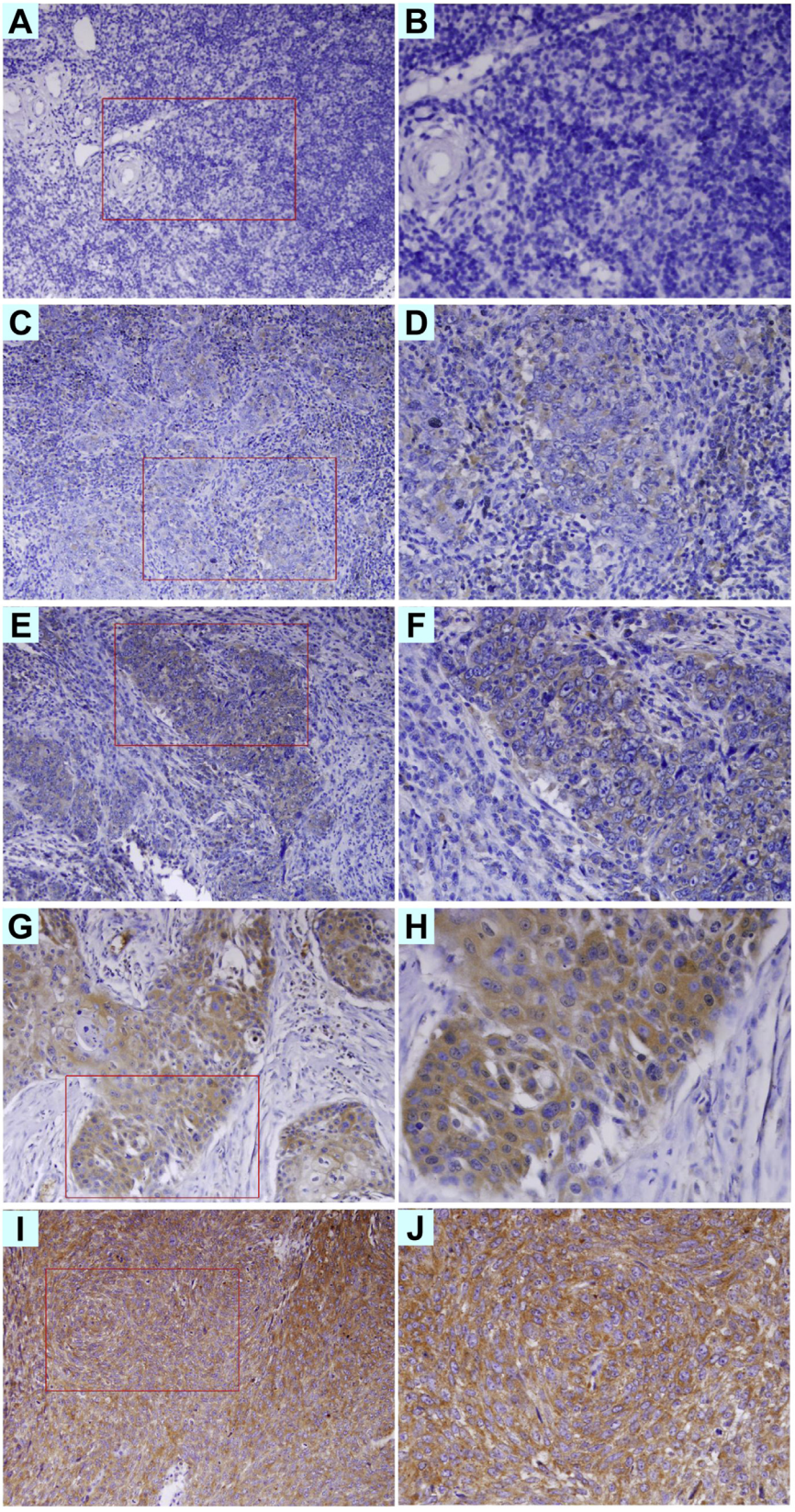

Fig. 2. The expression of SPHK1 in NPC tissues based on immunohistochemistry staining. (A and B) negative SPHK1 staining in non-tumorous nasopharyngeal epithelium tissue (negative control) (A) 200×, (B) 400×; (C–F) low SPHK1 expression in the NPC tissue (C and E) 200×, (D and F) 400×; (G–J) high SPHK1 expression level of in the NPC tissue (G and I) 200×, (H and J) 400×.

Table 1
Correlation between SPHK1 expression and clinicopathologic characteristics of NPC patients.

Characteristic	No.	SPHK1 expression		P^b
	(n = 142)	Low (n = 49)	High (n = 93)	
Gender				0.655
Male	101	36 (73.5%)	65 (69.9%)	
Female	41	13 (26.5%)	28 (30.1%)	
Age (years)				0.270
≤46	75	29 (59.2%)	46 (49.5%)	
>46	67	20 (40.8%)	47 (50.5%)	
Histology[a]				0.422
WHO type 1	7	1 (2.0%)	6 (6.5%)	
WHO type 2.1 + 2.2	135	48 (98.0%)	87 (93.5%)	
Clinical stage				0.001*
I–II	42	23 (46.9%)	19 (20.4%)	
III–IV	100	26 (53.1%)	74 (79.6%)	
T classification				0.029*
T1–T2	55	25 (51.0%)	30 (32.3%)	
T3–T4	87	24 (49.0%)	63 (67.7%)	
N classification				0.002*
N0–N1	92	40 (81.6%)	52 (55.9%)	
N2–N3	50	9 (18.4%)	41 (44.1%)	
Locoregional recurrence				0.018*
Yes	19	2 (4.1%)	17 (18.3%)	
No	123	47 (95.9%)	76 (81.7%)	
Distant metastasis				0.001*
Yes	28	2 (4.1%)	26 (28.0%)	
No	114	47 (95.9%)	67 (72.0%)	
Treatment				0.829
IMRT	62	22 (44.9%)	40 (43.0%)	
2D-CRT	80	27 (55.1%)	53 (57.0%)	

Abbreviations: SPHK1: sphingosine kinase 1; NPC: nasopharyngeal carcinoma; WHO: World Health Organization; IMRT: intensity-modulated radiotherapy; 2D-CRT: 2-dimensional conventional radiotherapy.
*Statistically significant ($p < 0.05$).
[a] 2005 World Health Organization (WHO) Classification: type 1, keratinizing squamous cell carcinoma; type 2.1, nonkeratinizing carcinoma, differentiated subtype; type 2.2, nonkeratinizing carcinoma, undifferentiated subtype.
[b] p-value from Chi-square or Fisher exact test.

3. Results

3.1. SPHK1 overexpression in NPC specimens

Quantitative RT-PCR assay was used to evaluate and compare the expression of SPHK1 in 30 fresh NPC biopsies and 10 non-tumorous nasopharyngeal epithelial tissues. The SPHK1 mRNA level in NPC tissues was significantly higher than that in the non-tumorous nasopharyngeal epithelial tissues (Fig. 1A). To confirm the overexpression of SPHK1 in NPC tissues, the protein level of SPHK1 in some tumor and non-tumorous tissues was evaluated by Western blotting. The Western blotting results were consistent with the qRT-PCR data (Fig. 1B). Taken together, SPHK1 was up-regulated in fresh NPC tissues.

3.2. Increased expression of SPHK1 in NPC tissues determined by IHC

We next examined the expression pattern of SPHK1 in 142 paraffin-embedded NPC biopsies and 10 non-tumorous nasopharyngeal epithelial tissues based on IHC. The representative results of IHC are shown in Fig. 2. We observed no or weak SPHK1 staining in these 10 cases of non-tumorous nasopharyngeal epithelial tissue (Fig. 2A and B). In contrast, positive staining of SPHK1 was observed in almost all among the 142 NPC biopsies. According to the SPHK1 staining index, 49 (34.5%) and 93 (65.5%) biopsies showed low (Fig. 2C–F) and high (Fig. 2G–J) expressions of SPHK1, respectively. In addition, SPHK1 was primarily localized in the cytoplasm of NPC cells (Fig. 2).

3.3. SPHK1 protein expression and the clinicopathological features of NPC

The relationship between SPHK1 expression and different clinicopathological features was shown in Table 1. Up-regulation of SPHK1 was significantly associated with clinical stages ($P = 0.001$), T classification ($P = 0.029$), N classification ($P = 0.002$), locoregional recurrence ($P = 0.018$), and distant metastasis ($P = 0.001$). No significant association was between SPHK1 expression and other clinicopathological features, such as patient age, gender, and WHO classification ($P > 0.05$, Table 1)

3.4. SPHK1 expression and NPC patients' survival

The five-year overall survival (OS) rate of the cohort of 142 NPC patients was 52% (Fig. 3A). The association between SPHK1 expression and survival of NPC patients was analyze based on Kaplan–Meier analysis and log-rank test. The results showed that NPC patients with high SPHK1 expression had significantly shorter overall survival than those with low SPHK1 expression (log-rank test: $P < 0.001$, Fig. 3B). We further evaluated the prognostic value of SPHK1 by comparing its expression in different subgroups of NPC patients stratified according to the clinical stages. Patients with high SPHK1 expression had significantly shorter overall survival than those with low expression of SPHK1 at either early stages (I + II) subgroup (n = 41; $P = 0.02$, log-rank; Fig. 3C) or advanced stages (III + IV) subgroup (n = 101; $P = 0.001$, log-rank; Fig. 3D), suggesting that SPHK1 is a valuable prognostic marker for NPC patients at all disease stages.

Univariate Cox proportional hazard regression analysis showed that clinical stages, T classification, N classification, locoregional recurrence, distant metastasis, and SPHK1 expression were significantly associated with overall survival of NPC patients ($P < 0.05$, Table 2). No significant association was found between SPHK1 expression and age, gender, treatment, and WHO classification. Based on the Cox proportional hazards model, multivariate analysis of the variables exhibited significant association with SPHK1 expression in the univariate analysis demonstrated that clinical stage, locoregional recurrence, distant metastasis, and SPHK1 expression were independent prognostic factors for NPC patients ($P < 0.05$, Table 2).

4. Discussion

Previous studies have demonstrated that SPHK1 was involved in cancer development and progression due to overexpression in several tumor types [27–29,32,35]. However, the role of SPHK1 in NPC is still not addressed. In the present study, we found that SPHK1 expression was significantly increased in the NPC biopsies compared with the non-tumorous nasopharyngeal mucosa tissues. We also found that high expression of SPHK1 protein correlated with tumor stages of NPC. Moreover, univariate and multivariate Cox regression analyses suggest that SPHK1 is an independent predictor for the prognosis of NPC patients.

It has been reported that ectopic expression of SPHK1 stimulated G1-Stransition, promoted cell growth and colony formation in soft agar, and induced malignant transformation in immunodeficient mice [21,24]. In addition, overexpression of SPHK1 improved cell proliferation, inhibited apoptosis, and promoted estrogen-dependent tumorigenesis in MCF-7 breast cancer cells [25]. On the contrary, inhibiting SPHK1 through small interfering RNA or SPHK1 inhibitors reversed the above mentioned oncogenic features

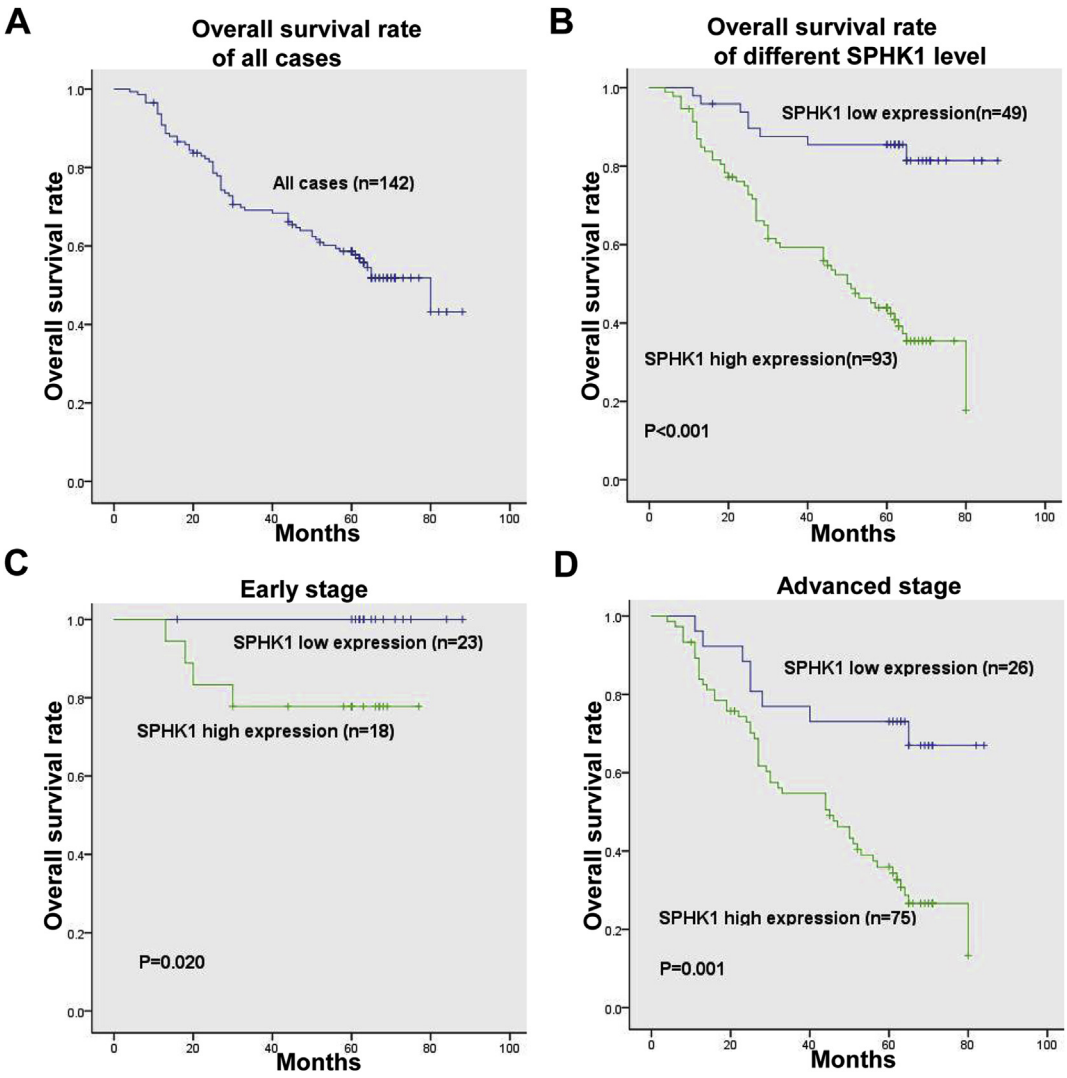

Fig. 3. The association between SPHK1 expression and NPC patient survival revealed by the Kaplan–Meier survival curve and log-rank test. (A) The five-year overall survival (OS) rate was 52% for 142 NPC patient; (B) The patients with high SPHK1 expression had significantly shorter survival than those with low SPHK1 expression ($P < 0.001$); (C) NPC cases were stratified through clinical stages. High SPHK1 expression was significantly associated with shorter OS ($P < 0.02$) in NPC patients at early stages (stages I + II); (D) High SPHK1 expression was significantly associated with shorter OS ($P < 0.001$) in NPC patients at advanced stages (stages III + IV).

[35]. Furthermore, it has been shown that SPHK1 served as a chemotherapy sensor and the combination of chemotherapy agents and SPHK1 inhibitor synergistically promoted the apoptosis of tumor cells [31,36,37]. In the present study, we found that SPHK1 expression was significantly increased in NPC tissues compared with non-tumorous nasopharyngeal epithelial tissues, suggesting that SPHK1 is involved in NPC carcinogenesis. Recently, increasing evidence suggest that SPHK1 functions as an oncoenzyme involved in carcinogenesis [18–20,24]. The possible mechanisms underlying the role of SPHK1 in the pathogenesis of NPC may include its enzymatic activity of up-regulating sphingosine-1-phosphate (S1P). It has been reported that a variety of factors, such as TNF-α, activated and promoted the translocation of SPHK1 from the cytosol to the plasma membrane and catalyzed the production of S1P by phosphorylating the 1-OH of sphingosine [38,39]. S1P is a potentially bioactive lipid involved in tumorigenesis [40]. S1P can either function as a second messenger intracellularly or induce cellular responses such as growth, survival, and migration of mammalian cells by extracellularly binding to G-protein-coupled receptors ($S1P_1$–$S1P_5$) [41], laying crucial roles in many human diseases including cancers [42].

It has been reported that SPHK1 overexpression serves as a prognostic marker for judging the survival of patients in several types of human cancers [27,28,32]. To this regard, we analyzed the relationship between SPHK1 staining and the clinicopathologic characteristics of the NPC patients and found that a significant correlation between SPHK1 expression and clinical stages and distant metastasis. Patients with high SPHK1 level exhibited shorter survival time, whereas those with lower SPHK1 expression survived longer. It is of note that there is a significant correlation between shorter overall survival times of patients and high SPHK1 expression in both the early stages (I + II) subgroup and the advanced stages (III + IV) subgroup, suggesting that SPHK1 may be a useful prognostic marker for NPC patients at all disease stages. Locoregional recurrence still represents a major cause of morbidity and mortality in advanced stages, and management of local failure remains a challenging issue in NPC [43]. However, the relationship between SPHK1 expression and tumor recurrence has not been reported before. In this study, we also find that high SPHK1 level associated with NPC patients' locoregional recurrence, with a locoregional recurrence rate of 18.3% in the high SPHK1 expression group in contrast to the 4.1% in the low SPHK1 expression group,

Table 2
Univariate and multivariate Cox regression analysis of different prognostic variables in NPC patients.

Variable	Subset	HR (95%) CI[a]	P^b
Univariate analysis (N = 142)			
SPHK1 expression	High versus Low	5.311 (2.522–11.183)	<0.001*
Gender	Female versus Male	0.769 (0.455–1.299)	0.334
Age (years)	>46 versus ≤46	1479 (0.902–2.424)	0.120
Histology	Type1 versus Type2	0.464 (0.200–1.077)	0.106
Clinical stage	I + II versus III + IV	8.089 (2.937–22.276)	<0.001*
T classification	T1+T2 versus T3+T4	3.045 (1.654–5.606)	<0.001*
N classification	N0+N1versusN2+N3	3.628 (2.194–5.998)	<0.001*
Locoregional recurrence	Yes versus No	2.112 (1.158–3.855)	0.023
Distant metastasis	Yes versus No	3.233 (1.917–5.450)	<0.001*
Treatment	IMRT versus 2D-RT	0.585 (0.349–0.981)	0.058
Multivariate analysis (N = 142)			
SPHK1 expression	High versus Low	2.666 (1.213–5.857)	0.015*
Clinical stage	I + II versus III + IV	3.824 (1.076–13.594)	0.038*
T classification	T1+T2 versus T3+T4	1.124 (0.539–2.347)	0.755
N classification	T1+T2 versus T3+T4	1.697 (0.978–2.946)	0.060
Locoregional recurrence	Yes versus No	2.244 (1.139–4.420)	0.019*
Distant metastasis	Yes versus No	2.331 (1.277–4.256)	0.006*

Abbreviations: HR: Hazard Ratio.
*Statistically significant ($p < 0.05$).
[a] 95% Wald Confidence Limits.
[b] p-value from Cox regression analyses.

suggesting the possibility of using SPHK1 as a predictor for patient locoregional recurrence.

In summary, our results indicate that high SPHK1 expression is associated with tumor progression and might represent a novel and useful prognostic marker for NPC patients. In addition, SPHK1 function as an oncoenzyme that is closely involved in carcinogenesis, however, the possible underlying mechanisms for its participation in tumor progression remain to be clarified yet, which might eventually lead to the development of a new anti-NPC strategy.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Conflicts of interest

None.

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