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From Cell Biology to Tissue Engineering

SPHK2 protein expression, Ki-67 index and infiltration of tumor-associated macrophages (TAMs) in human glioma

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Summary. Introduction. Sphingosine kinases (SPHKs), the Ki-67 index and tumor-associated macrophages (TAMs) are associated with diverse human malignancies, including glioma. SPHK2, a subtype of SPHKs, has not been assessed in glioma or correlated with the Ki-67 index or TAM infiltration. We tested the hypothesis that expression of SPHK2 correlates with the Ki-67 index and TAM infiltration in patients with glioma.

Materials and method. Western blot analysis was performed on protein lysates prepared from human astrocyte (HA) and glioma cell lines. Immunofluorescence was used to determine the subcellular location of SPHK2 protein in glioma cells. Next, immunohistochemistry was employed to analyze the correlations among SPHK2, Ki-67, CD68, and CD206 in 11 non-neoplastic brain tissues and 60 glioma tissues. All slides were evaluated under ×400 magnification, and the ratio of positively stained cells to the total number of cells was calculated for analysis.

Results. SPHK2, CD68 and CD206 were all increased in glioma tissues compared to non-neoplastic brain tissues, but there were no differences between WHO grades of glioma. Ki-67 was highest in WHO grade IV tumors and lowest in non-neoplastic brain tissues, and all between-group differences were statistically significant. Moreover, SPHK2 expression was positively correlated with the Ki-67, CD68 and CD206 indexes. Finally, the CD68 and CD206 indexes were both associated with the Ki-67 index.

Conclusion. SPHK2 protein expression, the Ki-67 index and TAM infiltration in human glioma tissue were reported in this study for the first time. SPHK2 was positively associated with TAM infiltration and glioma proliferation. Mechanistically, SPHK2 may promote glioma growth by stimulating TAMs to polarize M2-type macrophages.

Key words: Glioma, SPHK2, TAMs, Ki-67

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Introduction

Gliomas are the most common tumor in the brain and are among the deadliest of all cancers (Meyer, 2008). Despite aggressive therapy, most patients with glioblastoma multiforme (GBM) die within 2 years of diagnosis (Stupp et al., 2005; Clarke et al., 2010). A recent study reported that tumor-associated macrophages (TAMs) facilitate glioma proliferation and invasion, representing an alternative therapeutic target for glioma (Feng et al., 2015; Hambardzumyan et al., 2016). The macrophages/microglia that infiltrate cancer tissues are considered TAMs (Van Brocklyn et al., 2005). TAMs are generally thought to belong to the alternatively activated macrophage population (M2) because of their anti-inflammatory functions (Gordon, 2003). Expression of both CD68 and CD11b has been associated with the TAM phenotype, while CD206 has been associated with the M2-type TAM phenotype (Guo et al., 2016). Recent studies have also shown that the presence of M2 macrophages is associated with poor prognosis for

patients with various types of tumors, including glioma (Komohara et al., 2008). Hence, understanding the mechanism of interaction between TAMs and glioma may identify therapeutic strategies for glioma.

Sphingosine 1-phosphate (S1P) is an important bioactive lipid messenger that regulates cell proliferation and inhibits apoptosis (Mahajan-Thakur et al., 2017). Sphingosine kinase (SPHK) is the rate-limiting enzyme that maintains the level of S1P for cell survival and normal cell proliferation (Hatoum et al., 2017). There are two isoforms of SPHK, SPHK1 and SPHK2 (Dimasi et al., 2016). High SPHK1 expression has been reported in many tumors including glioma, and its expression was related to patient survival (Kapitonov et al., 2009; Funaki et al., 2017; Furuya et al., 2017; Huan et al, 2017; Shimizu et al., 2018). However, unlike SPHK1, there is much less research on SPHK2 in tumors, including glioma. Hence, we investigated the expression of SPHK2 in different grades of glioma and its function in glioma and the tumor microenvironment. Andreas Weigert et al. found that SPHK2-deficient tumor xenografts show impaired growth and failed to polarize macrophages towards an anti-inflammatory phenotype, although the mechanism was not explained in detail (Weigert et al., 2009). Thus, we sought to explore the relationship between SPHK2 and TAMs and the mechanism for how SPHK2 influences tumor cell growth and infiltration of different TAM phenotypes.

Ki-67 is a nuclear protein associated with cellular proliferation and is widely applied in routine clinical work (Inwald et al., 2013). The Ki-67 index is considered an important predictive parameter in human glioma and has been studied in relation to the development and progression of many tumors, including glioma (Su et al., 2017). However, little is known concerning the relationship between the Ki-67 index and TAM infiltration in glioma.

Thus, SPHK2 expression and its correlation with the Ki-67 index and TAM infiltration in human glioma have not been previously reported. We hypothesized that high SPHK2 expression correlates with tumor cell proliferation and TAM infiltration. The objectives of this study were as follows: I) contrast SPHK2 expression in gliomas with non-neoplastic brain tissues and assess its correlation with the Ki-67 index; II) determine TAM and M2-type TAM infiltration and evaluate the correlation with the Ki-67 index; III) compare TAMs in glioma and non-neoplastic brain tissues and evaluate the relationship between TAMs and the Ki-67 index; and IV) analyze the relationship between SPHK2 expression and TAM infiltration.

Materials and methods

Cell lines and cell culture

The human glioma cell lines U-87MG, U-251MG, U-373MG, SK-MG3, U-343MG, A172, LNZ-308, U-118MG, and U-138MG and normal human astrocytes (HA) were purchased from the China Academia Sinica Cell Repository (Shanghai). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37°C, 5% CO_2.

Tissue samples and slide preparation

All samples were discarded tissues from patients undergoing surgery at the Second People's Hospital of Shenzhen obtained under approval of the medical ethical committee. Twenty-three astrocytomas (WHO grade II), 19 anaplastic astrocytomas (WHO grade III) and 18 glioblastomas (GBMs) diagnosed by a certified pathologist were collected. In addition, 11 non-tumor brain samples were obtained from decompressive craniotomy for traumatic brain injury. Slides of 5-μm thickness were cut and subjected to SPHK2, Ki-67, CD68 and CD206 immunohistochemistry. Glioma samples were sub-divided into WHO grade II, WHO grade III and WHO grade IV groups according to their degree of malignancy to facilitate comparisons.

Western blot analysis

Cells were lysed in buffer containing phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were quantified colorimetrically using the BCA method. Then, 20-μg samples of total protein were electrophoresed on 10% Tris-glycine gels (Invitrogen) and electrotransferred to immunoblot PVDF membranes (BioRad). Membranes were probed with rabbit anti-human SPHK2 antibody (Abcam, 1:1,000) or mouse anti-human β-actin antibody (reference protein; Santa Cruz, 1:1,000) overnight at 4°C, followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz, 1:5,000) or anti-mouse IgG secondary antibody (Santa Cruz, 1:5,000) for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (ECL) reagents and visualized using the GeneGenius Imaging System.

Immunofluorescence

Cells were seeded in 24-well plates on glass coverslips with 500 μl medium overnight at 37°C, 5% CO_2. After the medium was removed, cells were rinsed with PBS three times and fixed with 4% formaldehyde in PBS at room temperature for 10 min followed by washing with PBS. Samples were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min in an ice bath. After BSA blocking, samples were stained with rabbit anti-human SPHK2 antibody (Abcam, 1:100) for 1 h and rinsed with PBS. Samples were incubated with TRITC-conjugated goat anti-rabbit IgG secondary antibody (Abcam, 1:1,000) for another hour, followed by washing with PBS. DAPI was used to stain cell nuclei, and images were obtained with a fluorescence microscope (OLYMPUS BX50/BXFLA/DP70; Olympus, Japan). Rabbit anti-human SPHK2 antibody (primary antibody) was replaced with normal rabbit

serum in negative control sections for these experiments.

Immunohistochemistry

Immunohistochemistry was performed for SPHK2, Ki-67, CD68 and CD206. Tissue slides were deparaffinised in xylene following dehydration with ethanol. Endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in methanol for 20 min at room temperature (RT). After antigen retrieval, slides were blocked with 5% BSA for 20 min at RT and then probed with rabbit anti-SPHK2 antibody (diluted 1:100; Abcam), mouse anti-Ki-67 antibody (diluted 1:100; BioLegend), mouse anti-CD68 antibody (diluted 1:100; Abcam) and rabbit anti-CD206 antibody (diluted 1:100; Abcam) at 4°C overnight. After washing, slides were incubated with biotinylated goat anti-rabbit immunoglobulin or horse anti-mouse immunoglobulin at RT for 1 h, stained with DAB complete peroxidase (Vector Laboratories), and counterstained with hematoxylin. Primary antibodies were replaced by normal rabbit serum in negative control sections for these experiments. Slides were evaluated under ×400 magnification, and the ratio of positively stained tumor cells to the total number of tumor cells was calculated for analysis.

Statistical analyses

All statistical analyses were performed with SPSS 18.0 software. Data are presented as the mean ± standard deviation (SD). The differences among/between sample groups were analyzed by one-way analysis of variance (ANOVA) or Student's t test. Pearson correlation analysis was used to determine the relationship among SPHK2, CD68, CD206 and Ki-67. Statistical

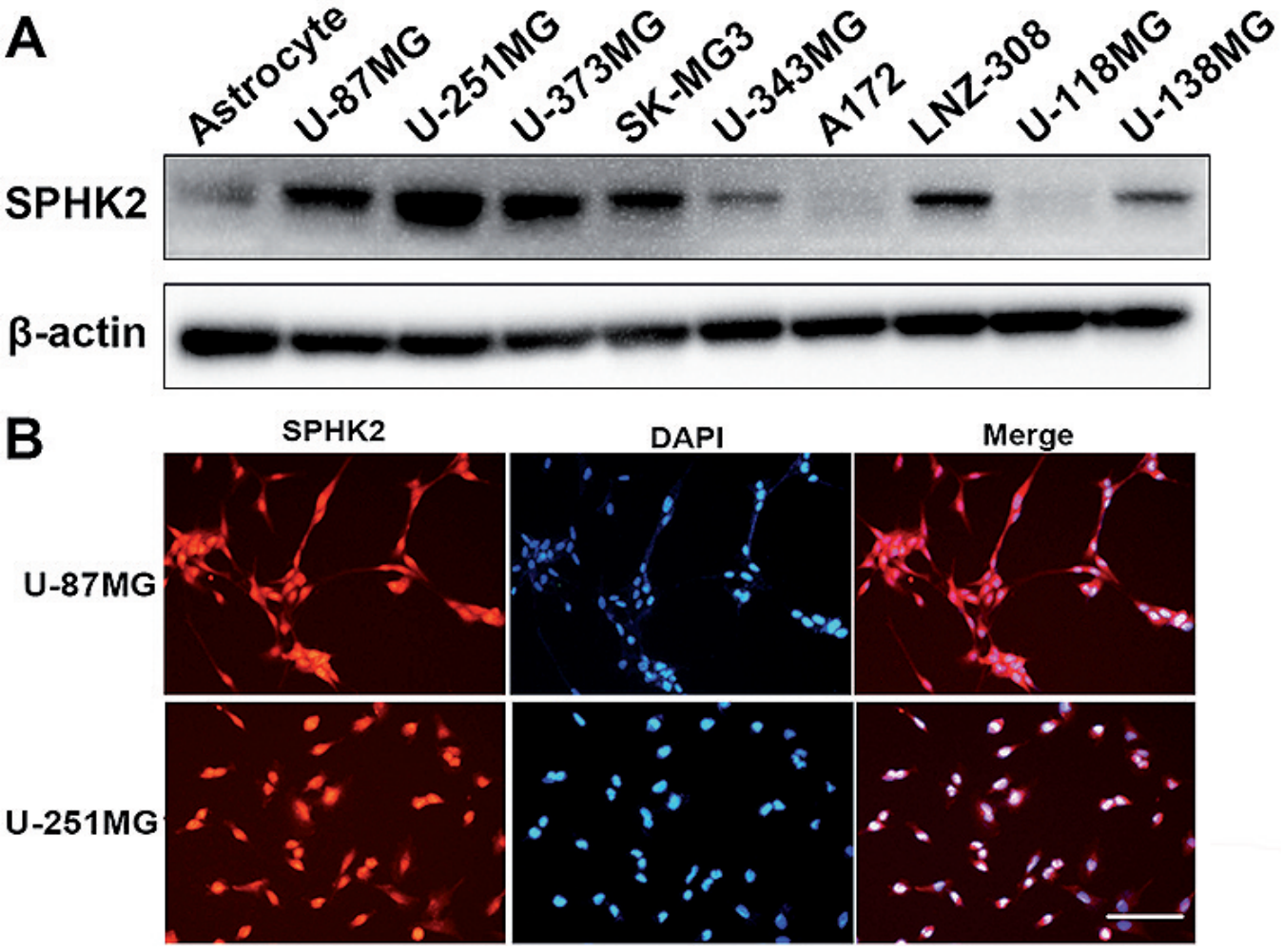

Fig. 1. Expression of SPHK2 in glioma cell lines. **A.** Expression levels of SPHK2 protein in nine glioma cell lines. **B.** Immunofluorescence detection of SPHK2. Nuclei of SPHK2+ cells are stained red, while those of the SPHK2- cells are stained blue by 4',6-diamidino-2-phenylindole (DAPI). Scale bars: 50 um.

significance was assigned at P<0.05, P<0.01 or P<0.001. All experiments with cell lines were performed at least three times with triplicate samples.

Results

SPHK2 protein is overexpressed in most human glioma cell lines and is located primarily in the nucleus

We used western blot to evaluate SPHK2 protein expression in all glioma cell lines. Of all included cell lines, only A172 and U-118MG cells showed no difference with the human astrocyte (HA) cell line, while the other 7 lines showed significantly higher SPHK2 expression than HA cells (Fig. 1A).

We further used immunofluorescence to detect the subcellular location of SPHK2 protein in the U-87MG and U-251MG cell lines. Our results indicated that SPHK2 expression in U-87MG and U-251MG cells was primarily located in the nucleus (Fig. 1B).

Clinicopathological characteristics of tumor tissue samples

Our samples included 23 WHO grade II gliomas (10 diffuse astrocytomas, 8 oligoastrocytomas and 5 oligodendrocytomas), 19 WHO grade III gliomas (7 anaplastic astrocytomas, 7 anaplastic oligodendrocytomas and 5 anaplastic oligoastrocytomas) and 18 WHO grade IV glioblastomas (Table 1).

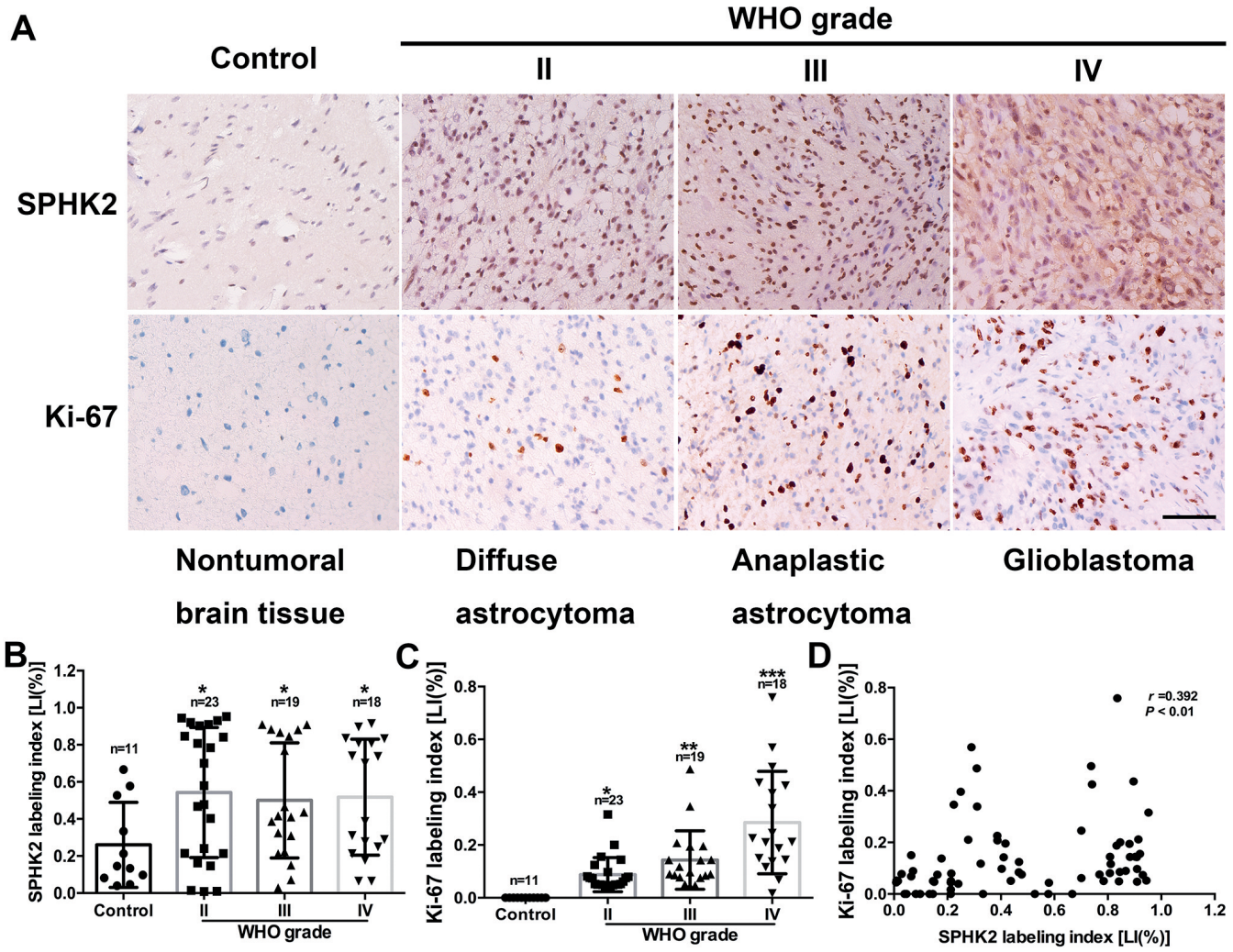

Fig. 2. SPHK2 is highly expressed in human glioma tissues and positively correlates with Ki-67. **A-D.** Eleven non-tumor brain samples (control), 23 astrocytomas (WHO grade II), 19 anaplastic astrocytomas (WHO grade III) and 18 glioblastomas (GBMs) were stained by immunohistochemistry to detect SPHK2 and Ki-67 expression. **A.** Representative images of different grades of glioma are shown. SPHK2 and Ki-67 expression in grade II, III and IV specimens was higher than in controls. The positive cell rates of SPHK2 **(B)** and Ki-67 **(C)** in glioma tissues were counted and analyzed. Data are shown as the mean ± SD; *P<0.05, **P<0.01, ***P<0.001. **D.** SPHK2 expression in gliomas positively correlated with Ki-67 expression (r=0.392, P<0.01). Scale bars: 50 um

SPHK2 labelling is increased in glioma tissues and positively correlates with Ki-67 labeling

SPHK2 expression was weak and present in only a small proportion of non-neoplastic brain tissues and was significantly higher in the majority of gliomas than in non-neoplastic brain tissues (P<0.05; Fig. 2A,B). However, the expression of SPHK2 was not significantly different between glioma grades (Fig. 2A,B).

To investigate the correlation between SPHK2 expression in gliomas and the proliferation index, we used immunohistochemistry to detect Ki-67 expression (proliferation index) in formalin-fixed paraffin-embedded (FFPE) specimens of glioma and non-neoplastic brain tissues. We discovered that Ki-67 expression in gliomas was higher than in controls (P<0.05~0.001) and that its expression was significantly increased with the elevation of glioma grades, being the highest in glioblastoma (P<0.01~0.001; Fig, 2,C). Moreover, SPHK2 expression in gliomas was positively correlated with Ki-67 expression (r=0.392, P<0.01; Fig. 2D).

Table 1. Histopathological subtypes and grades of the analysed gliomas.

Groups	Subtotal	Subtypes	Grades	Number of cases
WHO II	23	diffuse astrocytoma	WHO II	10
		oligoastrocytoma	WHO II	8
		oligodendrocytoma	WHO II	5
WHO III	19	anaplastic astrocytoma	WHO III	7
		anaplastic oligodendrocytoma	WHO III	7
		anaplastic oligoastrocytoma	WHO III	5
WHO IV	18	glioblastoma	WHO IV	18
Total	60			60

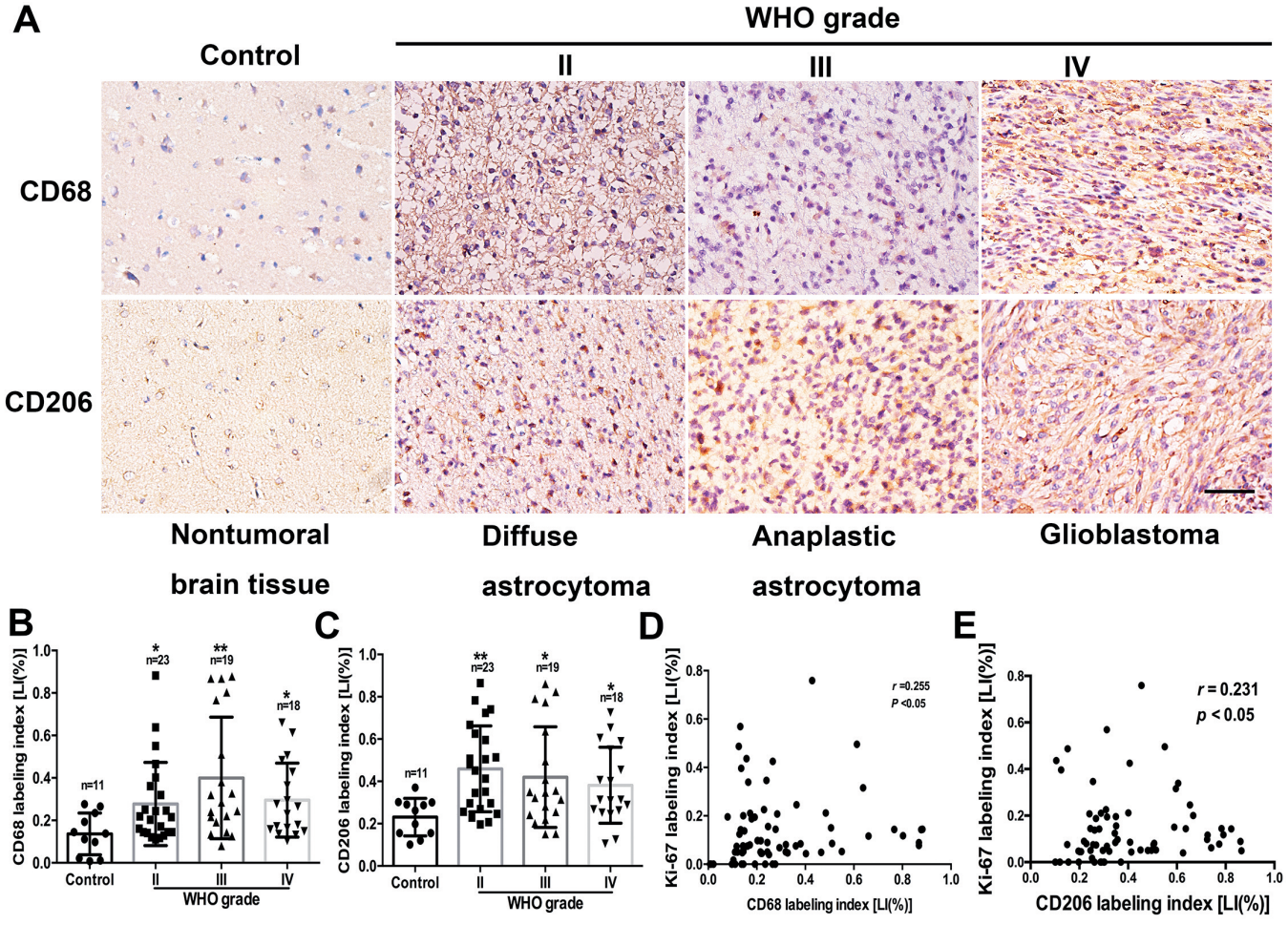

Fig. 3. CD68 (TAMs) and CD206 (M2-type TAMs) are highly expressed in human glioma tissues and positively correlate with Ki-67. **A-E.** Eleven non-tumor brain samples (control), 23 astrocytomas (WHO grade II), 19 anaplastic astrocytomas (WHO grade III) and 18 glioblastomas (GBMs) were stained by immunohistochemistry to detect CD68 and CD206 expression. **A.** Representative images of different grades of glioma are shown. CD68 and CD206 expression in grade II, III and IV specimens was higher than in controls. Positive cell rates of CD68 **(B)** and CD206 **(C)** in glioma tissues were counted and analyzed. Data are shown as the mean ± SD; *P<0.05, **P<0.01, ***P<0.001. **D, E.** CD68 and CD206 expression in gliomas positively correlated with Ki-67 expression (r=0.255, P<0.05; r=0.231, P<0.05). Scale bars: 50 um

These data revealed the expression of SPHK2 in gliomas and further indicate that SPHK2 overexpression is an important driver of proliferation of gliomas.

TAMs are activated in glioma tissues, and infiltration of TAMs (CD68) and M2-type TAMs (CD206) is positively correlated with proliferative activity in gliomas

To explore the relationships between glioma grades, proliferation index and infiltration of TAMs, we performed immunohistochemistry staining on FFPE specimens of glioma and non-neoplastic brain tissues. We found that infiltration of TAMs (CD68) and M2-type TAMs (CD206) was increased in gliomas with no significant differences between glioma grades ($P<0.05$~0.01; Fig. 3A,C). A correlation between the infiltration of TAMs (CD68), M2-type TAMs (CD206) and the Ki-67 index was also found in different grades of glioma ($r=0.255$, $P<0.05$; $r=0.231$, $P<0.05$; Fig. 3D,E).

Our results reveal that TAMs are activated in gliomas and that infiltration of TAMs and M2-type TAMs is positively correlated with proliferative activity in glioma. We conclude that infiltration of TAMs and M2-type TAMs contribute to the cellular proliferation in gliomas.

SPHK2 labelling positively correlates with CD68 (TAMs) and CD206 (M2-type TAMs)

The above results confirmed that expression of SPHK2 and infiltration of TAMs and M2-type TAMs were positively correlated with the proliferative activity of glioma cells. Next, we examined the relationship between SPHK2 expression and TAM or M2-type TAM infiltration. Our results showed that expression of SPHK2 positively correlated with CD68 (TAMs) and CD206 (M2-type TAMs) ($r=0.295$, $P<0.05$; $r=0.258$, $P<0.05$; Fig. 4A and 4B, respectively). These data suggest that SPHK2 overexpression results in TAM and M2-type TAM infiltration in gliomas.

Discussion

TAMs are important in cancer development (Bingle et al., 2002; He et al., 2014; Hu et al., 2016), although the true extent of microglial infiltration in human glioma was not, until recently, widely appreciated (Badie and Schartner, 2001; Graeber et al., 2002). TAMs have predominantly been studied based on detection of CD68, which recognizes macrophages on the M1 (classically activated) to M2 (alternatively activated) spectrum (Italiani and Boraschi, 2014; Schlereth et al., 2016). However, through detection of CD206, indicating the M2 phenotypic spectrum, a distinction can be made from the M1 spectrum (Kaku et al., 2014; Martinez and Gordon, 2014). Using these immunocytochemical markers, recent studies have emphasized the high numbers of microglia/macrophages consistently found within high-grade gliomas (Roggendorf et al., 1996; Sasaki et al., 1998, 2001, 2004; Nishie et al., 1999). However, the precise molecular mechanisms underlying this phenomenon have remained unclear.

S1P is an important bioactive lipid messenger that regulates cell proliferation and apoptosis. Sphingolipids are a diverse group of water-insoluble molecules that include ceramides, sphingoid bases, ceramide phosphates and sphingoid-based phosphates (Venkata et al., 2014). The dynamic balance between ceramide phosphates and sphingoid-based phosphates determines cell proliferation, invasion and apoptosis (Shida et al., 2008). SPHKs are the main limiting enzymes for sphingoid-base phosphates in cells and exist as two distinct isoforms, SPHK1 and SPHK2 (Liu et al., 2002; Oskeritzian et al., 2008;). SPHK1, an oncogenic kinase, is elevated in various human cancer types and is involved in tumor development and progression, including in glioma (Kapitonov et al., 2009). However, the biological functions of SPHK2 in glioma remain

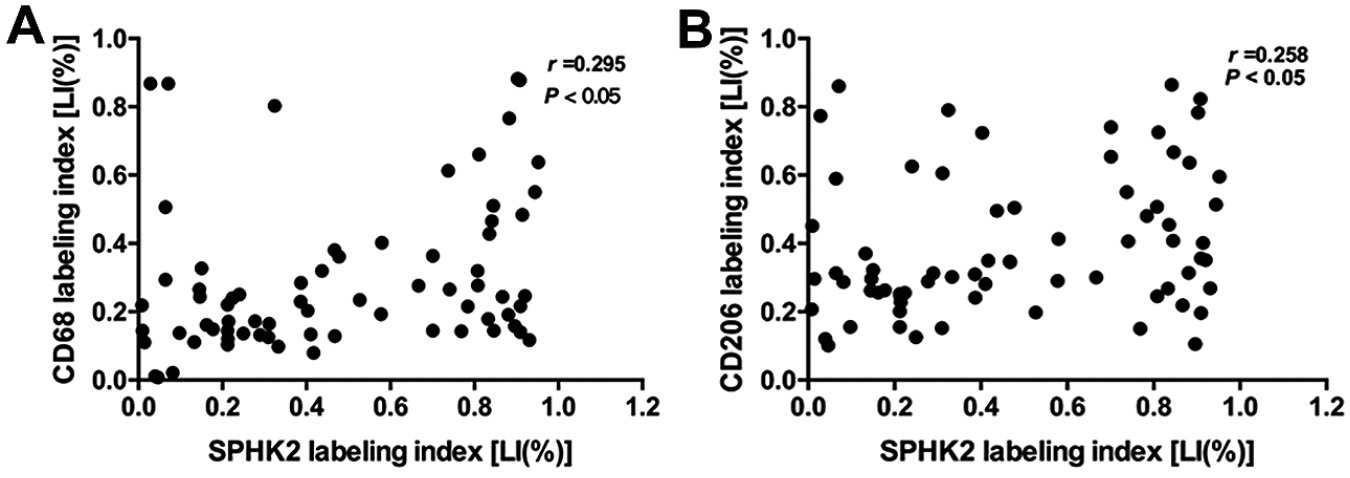

Fig. 4. CD68 **(A)** and CD206 **(B)** expression in gliomas positively correlated with SPHK2 expression ($r=0.295$, $P<0.05$; $r=0.258$, $P<0.05$).

unknown. Our data demonstrated that SPHK2 expression was significantly increased in glioma along with the Ki-67 index and that SPHK2 expression was positively correlated with Ki-67 expression (Fig. 2A-D). These findings confirm that SPHK2 is an oncogenic protein that may promote cell proliferation.

It is well known that Ki-67 expression is associated with the malignancy of various human tumors. Our study showed that Ki-67 expression in gliomas was higher than that in controls ($P<0.05\sim0.001$) and that its expression was significantly increased with the elevation of glioma grade, being the highest in glioblastoma ($P<0.01\sim0.001$; Fig. 2A,C). Our study further suggested that TAM and M2-type TAM infiltration was strongly correlated with proliferative activity in glioma cells based on the examination of CD68, CD206 expression and Ki-67 positivity in glioma tissues and non-neoplastic brain tissues.

Because Andreas Weigert et al. found SPHK2 to influence the growth of breast tumors though macrophage polarization, we further explored this relationship in glioma and found that SPHK2 expression was not only related to TAMs ($r=0.295$, $P<0.05$; Fig. 4A) but also related to M2-type TAMs ($r=0.258$, $P<0.05$; Fig. 4B) (Weigert et al., 2009). We detected increased TAM and M2-type TAM infiltration in glioma samples; however, there was no difference between low-grade glioma and high-grade glioma. These results were similar to the expression of SPHK2 observed in glioma.

TAMs play a key role in the formation of the tumor microenvironment and the processes of tumorigenesis, cell growth, invasion, metastasis, immune suppression, and the anti-tumor response (da Fonseca and Badie, 2013). M2-type macrophages support glioma development by inducing tumor cell proliferation, reducing anti-tumor functions and increasing expression of immuno-suppressive mediators (Kennedy et al., 2013). In our results, SPHK2 expression was positively correlated with TAMs, M2-type TAMs and the Ki-67 index. Hence, we conclude that SPHK2 promotes glioma cell proliferation by stimulating TAM differentiation into M2 cells. Detailed analysis of the underlying mechanisms should be examined in future studies.

In summary, we characterized SPHK2 expression, the Ki-67 index, and TAM and M2-type TAM infiltration in glioma. Our results demonstrated that numerous TAMs and M2-type TAMs were present in glioma tissues and that both TAMs and M2-type TAMs were significantly correlated with proliferative activity and SPHK2 expression in glioma. Higher expression of SPHK2 in gliomas than in non-neoplastic brain tissues indicates that SPHK2 may play a role in gliomagenesis. Furthermore, the relationship between SPHK2 expression, the Ki-67 index, TAMs, and M2-type TAMs indicates that SPHK2 may promote glioma cell proliferation by stimulating TAM development into M2 cells. The data in this study provide the groundwork for further investigation of the clinical significance of these findings for chemo-preventive and chemotherapeutic purposes in glioma.

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