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Oncogenic S1P signalling in EBV-associated nasopharyngeal carcinoma activates AKT and promotes cell migration through S1P receptor 3

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Running title: Aberrant activation of S1P signalling in nasopharyngeal carcinoma

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Abstract

Undifferentiated nasopharyngeal carcinoma (NPC) is a cancer with high metastatic potential that is consistently associated with Epstein-Barr virus (EBV) infection. In this study, we have investigated the functional contribution of sphingosine-1-phosphate (S1P) signalling to the pathogenesis of NPC. We show that EBV infection or ectopic expression of the EBV-encoded latent genes (EBNA1, LMP1 and LMP2A) can up-regulate sphingosine kinase 1 (SPHK1), the key enzyme that produces S1P, in NPC cell lines. Exogenous addition of S1P promotes the migration of NPC cells through the activation of AKT; shRNA knockdown of SPHK1 resulted in a reduction in the levels of activated AKT and inhibition of cell migration. We also show that the S1P receptor 3 (S1PR3) is over-expressed in EBV-positive NPC patient-derived xenografts and a subset of primary NPC tissues and that knockdown of S1PR3 suppressed the activation of AKT and the S1P-induced migration of NPC cells. Taken together, our data point to a central role for EBV in mediating the oncogenic effects of S1P in NPC and identify S1P signalling as a potential therapeutic target in this disease.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, Sphingosine-1-phosphate, S1P receptor

Introduction

Nasopharyngeal carcinoma (NPC) is a highly metastatic cancer that is exceptionally prevalent in Southern China and South East Asia [1, 2]. Approximately 75% of NPC patients present with late stage disease and approximately 30% of these patients develop distant metastases post therapy [1, 3]. Existing treatment for advanced disease is limited to concurrent chemoradiotherapy and unfortunately, due to the location of tumours in close proximity to many vital organs in the head and neck region, most survivors of NPC have an impaired health-related quality of life [4, 5]. A deeper understanding of the molecular basis of NPC is required to inform innovations in the therapeutic approach.

Undifferentiated NPC is invariably associated with Epstein-Barr virus (EBV) infection [6]. EBV latent gene expression in NPC is restricted to Epstein-Barr nuclear antigen 1 (EBNA1), latent membrane proteins (LMP 1 and LMP2), EBV-encoded RNAs (EBERs) and BamHI-A transcripts. Although the exact pathogenic role of EBV in NPC tumorigenesis remains to be fully elucidated, it is well established that the expression of these EBV latent genes results in the perturbation of many cellular processes, which leads to the development of NPC.

Given that distant metastasis is a major cause of death for NPC patients, we focused on the possibility that sphingosine-1-phosphate (S1P) signalling, a pathway that has been shown to promote cancer cell migration and invasion [7], could be involved in the pathogenesis of NPC. S1P is a bioactive lipid mainly produced by sphingosine kinase 1 (SPHK1) [8], an enzyme that is activated following phosphorylation at Ser225 by extracellular signal-regulated kinase 1/2 (ERK 1/2) and translocated to the plasma membrane to generate S1P [9]. High expression of SPHK1 was shown to be associated with poor prognosis in NPC [10], but the downstream biological

consequences of elevated SPHK1 in NPC cells have not been studied. The pro-oncogenic effects of S1P have been suggested to be mainly a consequence of alterations in the expression or function of one or more of five known G protein-coupled receptors (GPCR), S1PR1-5 [11]. We were particularly interested to explore if the aberrant activation of the SPHK1/S1P/S1PR axis could contribute to the migratory potential of NPC. We show for the first time that EBV infection enhances the expression of SPHK1 in NPC cells, and S1P induces NPC cell migration by activating AKT through S1PR3.

Materials and Methods

Cell lines, xenografts and tissue samples

The cell lines used in this study included: an immortalised nasopharyngeal epithelial cell line, NP69; eight NPC-derived cell lines, of which seven are EBV-negative (CNE1, CNE2, HK1, HONE1, SUNE1, TW01, TW04) and one of which is EBV-positive (C666-1). NPC cells stably infected with a recombinant EBV (Akata strain) or expressing individual EBV-encoded latent genes were generated, as previously described [12]. Four NPC patient-derived EBV-positive xenografts (xeno-2117, xeno-666, C15 and C17) were used in this study [13-15]. Paraffin-embedded archival NPC and non-malignant nasopharyngeal tissue samples were obtained from the tissue bank of Department of Anatomical and Cellular Pathology at the Prince of Wales Hospital, The Chinese University of Hong Kong (CUHK). The study protocol was approved by the Clinical Research Ethics Committee of the CUHK. All NPC samples were non-keratinising EBER-positive. The study protocol was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee, Hong Kong. The clinical characteristics of the NPC samples are listed in Supplementary Table 1.

Quantitative real-time PCR (Q-PCR)

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, UK) and subjected to reverse transcription using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Q-PCR was performed in triplicate using the ABI Prism 7000 Sequence Detection System and TaqMan Gene Expression Assays (SPHK1: Hs00184211_m1; S1PR3: Hs00245464_s1; Applied Biosystems, USA). GAPDH was amplified in the same reaction to serve as an internal control for normalization. Fold changes in gene expression were measured using the comparative threshold cycle method ($\Delta\Delta C_t$).

Western blotting

Cells were lysed in ice-cold NP40 lysis buffer [150mM NaCl, 1% IGEPAL® CA-630, 50mM Tris-HCl (pH 8.0)] containing protease inhibitors (cocktail set III; Calbiochem, Merck Millipore) and phosphatase inhibitors (Halt phosphatase inhibitor cocktail; Thermo Scientific, USA). Samples containing equal protein were separated under reducing conditions using SDS-PAGE and the proteins transferred to polyvinylidene difluoride membranes (Millipore, UK). The primary antibodies used in this study were anti-phospho-SPHK1 (1:1000; ECM Biosciences, USA), anti-total SPHK1 (1:1000; Cell Signaling Technology, USA), anti-phospho-AKT (Ser473; 1:1000; Cell Signaling Technology, USA), anti-total AKT (1:1000; Cell Signaling Technology, USA), anti- α -tubulin (1:10000; Sigma-Aldrich, USA) and anti- β -actin (1:5000; Sigma-Aldrich, USA). Bound antibodies were detected with peroxidase conjugated secondary antibodies and Enhanced Chemiluminescence reagents (Advansta, USA).

Transwell migration and invasion assays

Migration assays were carried out using fibronectin-coated (10 μ g/ml) polycarbonate filters (8 μ m pore size, Transwell, Corning). Cells were incubated in RPMI/0.5% FBS overnight and re-

suspended in migration buffer [RPMI containing 0.25 mg/ml fatty acid-free human albumin (Sigma)]. 1×10^6 cells were plated into the upper chamber and allowed to migrate for 19 hours in the presence or absence of S1P (Enzo Life Sciences) in the lower chamber. Migrated cells were stained with 0.1% crystal violet and counted in five random fields. For experiments using inhibitors of AKT (LY294002; 1 μ M; Merck, Germany) and S1PR1/S1PR3 (VPC23019; 1 μ M; Tocris Bioscience, USA), the inhibitors were added to the both lower and upper chambers and S1P (5 μ M) added to the lower chamber. For invasion assays, pre-coated invasion chambers (8 μ m pore size, BD BioCoat Matrigel) were used and cells allowed to invade for 48 hours.

Knockdown of SPHK1, AKT and S1PR3

Two SPHK1 shRNA lentiviral plasmids (pLKO.1/shSPHK1_S1, pLKO.1/shSPHK1_S2) and the non-targeting (control) shRNA (pLKO.1/NS) were kindly provided by Prof Chee-Onn Leong (International Medical University, Malaysia). Briefly, 293T cells were transfected with the lentiviral construct using polyethylenimine together with the packaging plasmid (psPAX2) and envelope plasmid (pMDG2). At 48 hour post-transfection, the lentivirus-containing supernatant was centrifuged and filtered through a 0.45 μ m syringe filter. The viral supernatants and polybrene (8 μ g/ml) were then added to plates cultured with NPC cells and incubated for 16 hours. The virus-containing media were then removed and the cells cultured in RPMI/10% FBS for an additional 48 hours before selecting the transfected cells with 0.5-1 μ g/ml puromycin. To knockdown AKT and S1PR3, pre-designed RNAi SMART pool reagents (AKT1: L-003000-00; S1PR3: LU-005208-00) were obtained from Dharmacon (Chicago, IL) and used at 25 nM. 5×10^4 cells per well were seeded into 6-well plates, cultured for 16 hours and then transfected with the relevant siRNA using Oligofectamine (Invitrogen, UK).

Expression of constitutively active AKT

The vector carrying a myristoylated form of AKT, 1036 pcDNA3 Myr HA Akt1 (Addgene plasmid # 9008) [16], was used to express constitutively active AKT. Cells at 50% confluency were transfected with the 1036 pcDNA3 Myr HA Akt1 or the empty vector at a 3:1 FuGENE HD transfection reagent (Promega, USA):DNA ratio. Cells were used in Transwell migration assays 24 hours post-transfection.

RNA in-situ hybridization

SIPR3 mRNA expression in the NPC specimens was detected by RNAscope 2.0 assay with a SIPR3-specific probe (Advanced Cell Diagnostics, Hayward, CA, USA). FFPE tissue and cell block sections (5 µm) were deparaffinized in xylene and then dehydrated in a series of ethanol. The SIPR3-specific probes were hybridized to the sections, followed by incubation with preamplifier, amplifier and label probes. The chromogenic detection procedures were performed according to the manufacturer's instructions. SIPR3 expression was assessed by assigning a proportion score and an intensity score. The proportion score relates to the percentage of tumour cells with a positive signal (0-100). The intensity score represents the average amount of signals in the tumour cells (0, none; 1, weak; 2, intermediate; 3, strong). The SIPR3 expression score was the product of the proportion and intensity scores, ranging from 0 to 300.

Analysis of microarray data

Microarray raw data, GSE12452 and GSE34573, were downloaded from GEO and re-analyzed to identify genes differentially expressed between micro-dissected NPC tissues and normal nasopharyngeal epithelium. Probe level quantile normalisation [17], RMA (robust multi-array analysis) [18] and MAS5 detection analyses were performed using the affy package of the Bioconductor project (<http://www.bioconductor.org>). P values and fold changes were calculated

using limma [19]. Affymetrix “HG-Focus.na35.annot.csv” was used for probe set annotation. Probe sets with “Negative Strand Matching Probes” were removed. Differentially expressed genes were identified using the criteria of fold change > 1.5 and p value < 0.05 .

Statistical analyses

All statistical analyses were performed using SPSS 23.0 for Windows (IBM Inc., USA) or GraphPad Prism Version 5 (GraphPad Software Inc., USA). For Q-PCR and migration/invasion assays, statistical differences between experimental groups were evaluated by Student’s t-test or one-way analysis of variance (ANOVA)/Dunnett’s test. A receiver operating characteristic (ROC) curve was constructed using S1PR3 expression scores to select the best cut-off point (maximum sensitivity and specificity) that is relevant to the patient survival [20]. NPC cases were group into cases with low (score 0-45) or high (score 46-200) expression of S1PR3. Kaplan-Meier survival analysis was used to correlate survival rates with S1PR3 expression and the survival probability differences were compared by log-rank test. p-values < 0.05 were regarded as significant.

Results

EBV infection of NPC cells increases the expression of SPHK1

Given that SPHK1, the key enzyme that produces S1P, has been shown to be commonly expressed in primary NPC tissues [10], we opted to investigate whether EBV infection could modulate SPHK1 levels in NPC cells. We first compared SPHK1 mRNA expression in eight NPC cell lines with that in the immortalised nasopharyngeal epithelial cell line NP69. Among the three NPC cell lines that expressed significantly higher levels of SPHK1, we had noted that C666-1, the only EBV-positive NPC cell line, showed an exceptionally high expression of SPHK1 (Figure 1A). We subsequently found that SPHK1 mRNA was significantly elevated in six out of seven NPC cell lines stably infected with a recombinant EBV (Akata strain) ($p < 0.01$; Figure 1B). After first

validating the specificity of antibodies against total and phosphorylated SPHK1 proteins (Supplementary Figure 1), we showed that while the increased expression of the total SPHK1 protein was confirmed in three representative EBV-infected NPC cell lines, the levels of phosphorylated SPHK1 protein were also increased in these cell lines (Figure 1C). Next, we investigated which EBV gene(s) were responsible for this effect. To do this, we examined SPHK1 expression in HONE1 cells individually transfected with EBNA1, LMP1 or LMP2A, all of which are expressed in NPC in vivo. We found that both the mRNA and protein levels of SPHK1 were up-regulated by all the three EBV latent genes in HONE1 cells, with the most marked effect in LMP2A-expressing cells (Figure 1D). We conclude that EBV infection can up-regulate the expression of SPHK1, hence high levels of S1P are likely to be present in EBV-positive NPC cells.

S1P enhances the migration of NPC cells

Next, we used Transwell assays to investigate the effects of S1P on the migration of NPC cells using three representative NPC cell lines (TW04, HONE1 and SUNE1). We found that the exogenous addition of S1P significantly increased the migration of all three cell lines ($p < 0.001$; Figure 2A). ~~Given that C666-1 cells do not migrate in Transwell assays, even in the presence of 10% FBS or S1P, we performed wound healing assays using charcoal-stripped FBS. Similarly, S1P enhanced C666-1 cell migration (Supplementary Figure 2).~~ S1P also promoted the invasion of SUNE1 cells (Supplementary Figure 3). We next measured migration in HONE1 cells following their transduction with two different shRNAs targeting SPHK1 (shSPHK1_S1 or shSPHK1_S2), or with a non-targeting shRNA (NS), as a control. ~~Although C666-1 expressed the highest levels of SPHK1 among all the NPC cell lines analysed (Figure 1A), they do not migrate under serum free conditions, even in the presence of S1P (data not shown). Therefore, HONE1 cells were selected for these experiments.~~ Reduced expression of both SPHK1 mRNA and protein in transfected HONE1 was confirmed by Q-PCR and western blot analyses, respectively (Figure 2B). In line with the data

that S1P induced NPC cell migration, knockdown of SPHK1 expression suppressed the migration of HONE1 cells ($p < 0.001$; Figure 2C).

S1P induces NPC cell migration through activation of AKT

We next investigated the mechanism of S1P-induced migration of NPC cells. We focussed on the AKT pathway because AKT has been shown to be a central player in mediating the oncogenic effects of S1P and its activation promotes cancer cell migration in various solid tumour types [7]. Secondly, activation of the PI3K/AKT pathway is a common feature of NPC and this pathway is stimulated by the EBV-encoded LMP1 or LMP2A genes to promote the migration and invasion of NPC cells [21]. We found that the addition of exogenous S1P activated AKT in both HONE1 and TW04 cells (Figure 3A). To determine if the activation of AKT by S1P was responsible for the increased migration, we treated NPC cells with a PI3K/AKT inhibitor, LY294002. After first confirming the inhibitory effects of LY294002 on AKT phosphorylation (Supplementary Figure 4), we showed that treatment of HONE1 and SUNE1 with LY294002 led to a significant reduction in cell migration in the presence of S1P ($p < 0.001$; Figure 3B). Similarly, knockdown of AKT with a pool of four siRNAs targeting AKT significantly suppressed the migration of both HONE1 and SUNE1 cell lines ($p < 0.001$; Figure 3C). In keeping with these observations, the knockdown of SPHK1 also resulted in a significant reduction in the levels of activated AKT in HONE1 cells (Figure 3D). Furthermore, the expression of a constitutively active AKT was able to rescue the repressive effects of SPHK1 knockdown on cell migration ($p < 0.001$; Figure 3E). Taken together, our results show that S1P induces the migration of NPC cells through the activation of AKT.

Over-expression of S1PR3 in NPC

We next explored which S1P receptors might be responsible for mediating the S1P-induced migration of NPC cells. Re-analyses of two published microarray datasets, GSE12452 [22] and

GSE34573 [23], revealed significant and consistent up-regulation of S1PR3 mRNA expression in micro-dissected NPC cells compared to normal epithelium ($p < 0.05$; Figure 4A). Expression of the other four S1P receptor genes was either not significantly different or not consistently altered in both datasets (Supplementary Table 2). We attempted to examine the expression and cellular localisation of S1PR3 protein in primary NPC tissues using immunohistochemistry, however, none of the three anti-S1PR3 antibodies tested were suitable. Therefore, we opted to use sensitive RNA in situ hybridization (RNAscope) to examine the expression of S1PR3 in C666-1 cells, four EBV-positive NPC patient-derived xenografts (xeno-666, xeno-2117, C15 and C17), an independent cohort of 21 formalin-fixed, paraffin-embedded primary NPCs and nine non-malignant nasopharyngeal samples. We showed that C666-1 cells, xeno-666 and C15 demonstrated strong staining for S1PR3, while weak expression was observed in xeno-2117 and C17 (Figure 4B). In tissue samples, normal surface epithelium and epithelium adjacent to the carcinoma were consistently negative, whereas S1PR3 expression was detected in 13 of 21 (62%) of the primary NPCs examined (Figure 4C).

S1P mediates migration of NPC cell by activating AKT through S1PR3

Having shown that S1PR3 is over-expressed in primary NPC, we next wanted to determine if this receptor was involved in the activation of AKT and increased migration of NPC cells. To do this, we first treated two NPC cell lines that express S1PR3 (HONE1 and SUNE1; Supplementary Figure 5) with a S1PR1/S1PR3 antagonist, VPC23019. We found that VPC23019 decreased the migration of both cell lines in the presence of S1P ($p < 0.001$; Figure 5A). Next, we transfected SUNE1 cells with a pool of four siRNAs targeting S1PR3 and the reduced expression of S1PR3 was confirmed by Q-PCR analysis (Figure 5B). We observed that knockdown of S1PR3 reduced the levels of activated AKT (Figure 5C) and partially suppressed the migration of SUNE1 cells in the presence of S1P ($p < 0.001$; Figure 5D). To further confirm these effects, we co-transfected SUNE1 cells with a constitutively active AKT and S1PR3 siRNAs. Expression of a constitutively

active AKT restored the migration of these cells ($p < 0.001$; Figure 5E). We conclude that S1P enhances the migration of NPC cells via the activation of AKT by S1PR3.

Discussion

S1P signalling affects many processes involved in carcinogenesis and there is now a growing appreciation that the oncogenic effects of S1P are dependent on the enzymes that generate or degrade S1P, and/or alterations in the expression of S1P receptors [7, 11]. Thus, the SPHK/S1P/S1PRs axis has become an attractive therapeutic target in cancer. Two functional SPHK isoforms, SPHK1 and SPHK2, have been identified in humans [24]. Whilst the role of SPHK2 in carcinogenesis remains to be fully explored, the tumour-promoting role of SPHK1 is well acknowledged [25]. Indeed, over-expression of SPHK1 has been reported in various types of cancer, including NPC [10, 25]. A wide variety of stimuli, including growth factors, GPCR agonists, cytokines, phorbol esters, and vitamin D3, can increase SPHK1 levels [26]. In the present study, we have identified a novel role for EBV in stimulating the expression and activation of SPHK1 in NPC cell lines. Although the mechanisms by which the EBV-encoded genes up-regulate the transcription of SPHK1 are yet to be determined, LMP1 and LMP2A are known to activate the ERK-MAPK pathway in NPC [27] and this, in turn, would likely result in the phosphorylation of SPHK1.

High expression of SPHK1 increases the production of S1P and elevated levels of S1P promotes a number of malignant phenotypes associated with cancer cells, including proliferation, migration, invasion and angiogenesis [7]. Focussing on the migratory phenotype, our data convincingly show that S1P promotes the migration of NPC cells through the activation of AKT, and these effects were reversed by the knockdown of SPHK1. We further show that S1P activates AKT specifically via S1PR3 to promote the migration of NPC cells. Significantly, the PI3K/AKT

signalling pathway is frequently activated in NPC [21]. There is large body of evidence to show that S1P-induced migration of cancer cells is dependent upon the expression pattern of S1P receptors [28]. S1PR1 and S1PR3 have been shown to stimulate cell migration by coupling to G_i to activate Rac through PI3K; although S1PR2 is generally thought to inhibit cell migration via $G_{12/13}$, we recently showed that S1PR2 contributes to the S1P-induced migration of oral cancer cells [29]. The involvement of S1PR3-mediated AKT activation in S1P-induced migration has previously been inferred indirectly, in a limited number of studies. For example, S1P activated AKT in endothelial cells, ovarian cancer cells and thyroid cancer cells and treatment of these cells with LY294002 or wortmannin suppressed S1P-induced migration in a G_i -dependent manner [30-32]. Although these studies used the S1PR1/3 inhibitor, VPC23019, to reveal the possible involvement of S1PR1 and S1PR3 in mediating these effects, it was not clear which receptor was precisely responsible. Similar observations were reported in breast cancer cells, but here, S1P induced migration was mediated through G_{α_q} [33]. Although knockdown of G_{α_q} or S1PR3 inhibited AKT phosphorylation, the authors did not provide a direct role for S1PR3 in mediating the migration of breast cancer cells through AKT. Therefore, we are the first to demonstrate that S1PR3 stimulates cell migration via the activation of AKT.

The pro-migratory feature of S1PR3 was also reported to be mediated through the accumulation of phosphorylated ERK1/2 into membrane ruffles/lamellipodia and the nucleus of the breast cancer cell line, MCF-7 [34]. In the present study, S1P induced the phosphorylation of ERK in NPC cells; however, knockdown of SPHK1 did not affect the levels of phosphorylated ERK (data not shown). Nonetheless, we cannot rule out the possibility that S1PR3 can also mediate the S1P-induced migration of NPC cells through mechanisms other than AKT activation. S1P/S1PR3 signalling has been shown to promote cancer cell migration/invasion by up-regulating the expression of epidermal growth factor receptor (EGFR), hypoxia-induced factor-1 (HIF-1) or C-

reactive protein (CRP) [35-37]. The identification of effector(s) downstream of S1P/S1PR3/AKT signalling that stimulate the migration of NPC cells warrants further investigation. Further, we noted that knockdown of S1PR3 did not completely suppress the S1P-induced migration in SUNE1 cells. There are two possible reasons to explain these observations; one is the incomplete knockdown of S1PR3, and the second, another S1P receptor(s) might also be involved. Given that the up-regulation of S1PR5 was demonstrated in one of the microarray studies (Table S2), the involvement of this receptor in S1P-induced migration of NPC cells cannot be ruled out. Although the role of S1PR5 in tumorigenesis is still uncertain, it remains a possibility that by coupling to G_i, S1PR5 can trigger Rac that lead to an increase in NPC cell migration.

It has been shown that SPHK1 and S1PR3 function in an amplification loop to promote the migration of breast cancer cells [34]. However, we did not observe any consistent changes in S1PR3 expression following either knockdown of SPHK1 or EBV infection of NPC cells (data not shown). Since C15 is the only NPC xenograft that expresses LMP1, the high expression of S1PR3 in C15 prompted us to investigate whether LMP1 could modulate S1PR3 expression. However, of the 19 tumours in this series that were also stained for LMP1, we did not observe a correlation between S1PR3 and LMP1 expression (data not shown). Therefore, the mechanisms that regulate S1PR3 expression in NPCs remain to be determined and our data suggest that the expression of SPHK1 and S1PR3 in NPC cells are regulated by different mechanisms. Irrespective of these differences, high levels of SPHK1 in NPCs have been correlated with poor patient prognosis [10] and high expression of S1PR3 in the present study is associated with a worse overall survival in NPC patients ($p < 0.05$; Supplementary Figure 6), data that are in line with those of Watson and colleagues who reported an association between high S1PR3 expression and shorter disease-specific survival in breast cancer patients [38].

In summary, we report for the first time that S1P is likely produced by NPC cells through the up-regulation of SPHK1 following EBV infection, and that S1P contributes to a migratory phenotype of NPC cells by activating AKT through S1PR3. Our data highlight the potential of targeting of SPHK1/S1P/S1PR3 axis in NPC patients.

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Author contributions

LFY, ICP and PGM conceived and designed the study; HML, KWL, **GTYC**, MHI performed experiments; WW performed microarray data analyses; SWT and CWD produced transfected cell lines; ICP, PGM and CWD critically reviewed the manuscript and contributed intellectual opinion; LFY and ICP supervised the study. LFY and HML wrote the manuscript. All authors reviewed the manuscript.

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Figure legends

Figure 1. EBV infection up-regulates the expression of SPHK1. (A) Compared to an immortalised nasopharyngeal epithelial cell line (NP69), SPHK1 levels were significantly increased in three (C666-1, HONE1 and TW01) out of eight NPC cell lines examined. Among these, C666-1 expressed the highest levels of SPHK1. (B) Q-PCR analysis showed that the expression of SPHK1 was significantly up-regulated in six (HK1, CNE1, SUNE1, HONE1, CNE2, TW01) out of seven NPC cell lines stably infected with an Akata-derived recombinant EBV. Data are expressed as the relative expression between the cells infected with EBV and their respective controls (normalised to 1). (C) Western blot analysis showed that the protein levels of SPHK1 (both total and phosphorylated proteins) were increased in three NPC cell lines (HK1, CNE1 and SUNE1) following EBV infection. (D) Q-PCR and western blot analyses showed that both the mRNA and protein levels of SPHK1 were higher in HONE1 cells expressing specific EBV latent genes (EBNA1, LMP1 or LMP2A) compared with the vector control cells. *** $p < 0.001$.

Figure 2. S1P promotes NPC cell migration. (A) TW04, HONE1 and SUNE1 cells were allowed to migrate through fibronectin-coated membranes in the absence or presence of S1P (1 or 5 μ M). S1P significantly enhanced the migration of all three cell lines. (B) HONE1 cell stably transduced with two independent shRNAs (shSPHK1_S1 or shSPHK1_S2) or a non-targeting shRNA (NS). Upper left panel: Q-PCR analysis confirmed the reduced levels of SPHK1 mRNA in SPHK1 shRNA-transfected cells. The expression of SPHK1 in NS (control) cells was normalised to 1. Lower left panel: A representative Western blot showing decreased expression of both total and phosphorylated SPHK1 proteins in cells transfected with SPHK1 shRNAs. Densitometric data (right panels) are expressed as the mean relative density (normalised to α -tubulin) \pm SD from three independent experiments. (C) SPHK1 knockdown significantly suppressed migration of HONE1 cells. Data for the migration assays are expressed as the mean percentage of cells migrating \pm SD and the results are expressed relative to the migration of the control cells (=100%). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. S1P promotes migration of NPC cells by activating AKT. (A) Western blot analyses showed that AKT was activated (assessed by AKT phosphorylation) in HONE1 and TW04 cells following exogenous addition of 5 μ M S1P. (B) Transwell migration assays of HONE1 and SUNE1 cells in the absence or presence of S1P (5 μ M) in the lower chamber and a PI3K/AKT inhibitor (LY294002; 1 μ M) in both the upper and lower chambers. Treatment with LY294002 significantly inhibited the S1P-induced migration of these two NPC cell lines. (C) Knockdown of AKT using a pool of four siRNAs targeting AKT significantly suppressed the migration of both HONE1 and SUNE1 cell lines. (D) Upper panel: Western blotting analysis showed that knockdown of SPHK1 in HONE1 cells using two independent shRNAs (shSPHK1_S1 or shSPHK1_S2) suppressed the activation of AKT compared to the control (NS). Densitometric data (lower panels) are expressed as the mean relative density (normalised to α -tubulin) \pm SD from three independent experiments. (E) Transfection of a constitutively active AKT in SPHK1-knockdown HONE1 cells restored the migration of these cells. Data for the migration assays are expressed as mean percentage of cells

migrating \pm SD and the results are expressed relative to the migration of control cells (=100%). * p <0.05; ** p <0.01; *** p <0.001.

Figure 4. Over-expression of S1PR3 in NPC. (A) Two microarray datasets (GSE12452 and GSE34573) showed that S1PR3 mRNA was significantly upregulated in micro-dissected NPC cells compared with normal epithelium (p <0.05). (B) RNAscope analysis of S1PR3 expression in NPC-derived EBV-positive cell line and xenografts. [a-c] C666-1, xeno-666 and C15 demonstrating strong S1PR3 signals (red arrows) (magnification: x400); [d, e] Weak expression of S1PR3 was detected in xeno-2117 and C17. (C) RNAscope analysis confirms over-expression of S1PR3 in primary NPC tissues. [a, b] Two separate examples of normal epithelium demonstrating negative staining. Representative NPC cases showing [c] negative, [d] weak and [e, f] strong expression of S1PR3 in the carcinoma (red arrows) (magnification: X400).

Figure 5. S1P induces NPC cell migration through AKT activation via S1PR3. (A) Transwell migration assays of HONE1 and SUNE1 cells in the absence or presence of S1P (5 μ M) in the lower chamber and a S1PR1/S1PR3 antagonist (VPC23019; 1 μ M) in both the upper and lower chambers. S1P-induced migration was markedly suppressed in both cell lines following treatment with VPC23019. (B) SUNE1 cells were transfected with 25nM of a pool of siRNAs targeting S1PR3 (siS1PR3) or non-targeting random siRNAs (NT) for 48 and 72 hours. QPCR-analysis confirmed the knockdown of S1PR3 in SUNE1 cells. SUNE1 cells transfected with NT siRNAs were normalized to 1. (C) The levels of phosphorylated AKT were reduced in SUNE1 cells following S1PR3 knockdown. (D) Migration of SUNE1 cells following S1PR3 knockdown in Transwell assays in the absence or presence of S1P (5 μ M). Inhibition of S1PR3 significantly suppressed the migration of SUNE1 cells accompanied by a decrease in AKT phosphorylation. (E) Transwell assays of transient transfection of S1PR3-knockdown SUNE1 cells with a constitutively active AKT or the empty vector. The expression of a constitutively active AKT significantly restored the migration of S1PR3-knockdown SUNE1 cells. Data for Transwell migration assays are expressed as mean percentage of cells migrating \pm SD relative to migration of the control cells (=100%). ** p <0.01; *** p <0.001.

Legends for Supporting Information

Figure S1 Validation of the specificity of antibodies against total or phosphorylated SPHK1 protein. A distinct band corresponding to the predicted molecular weight of SPHK1 protein (45 kDa) was evident in HEK293 cells transfected with a SPHK1 construct (pCMV6_XL4/SPHK1).

Figure S2 S1P promotes migration of C666-1 cells in wound healing assays. The effect of S1P on C666-1 cell migration was examined in wound healing assays using charcoal-stripped FBS (Biowest, France). Left panel: Representative images showing enhanced migration of C666-1 cells in the presence of S1P at 24 hour. Right panel: Images were analysed using WimScratch (Wimasis Image Analysis) and the data are expressed relative to the wound area at 0 hour (=100%). * $p < 0.05$.

Figure S3 S1P enhances invasion of SUNE1 cells *in vitro*. The invasive ability of SUNE1 was examined in pre-coated invasion chambers (8 μm pore size, BD BioCoat Matrigel) in the presence of S1P in the lower chamber.

Figure S4 Confirmation of the inhibition of AKT activation following treatment with a PI3K/AKT inhibitor, LY294002. Western blotting analyses of SUNE1 cells treated with 1 μM LY294002 or the vehicle control for 19 hours.

Figure S5 Expression profiles of S1P receptors in HONE1 and SUNE1 cells. All five receptors were detected in both cell lines.

Figure S6 Kaplan Meier survival analysis revealed higher expression of S1PR3 correlated with poor patient survival ($p < 0.05$).

Table S1 Clinical characteristics of primary NPC samples.

Table S2 Summary of the mRNA expression of five S1P receptors in NPC cells. For each probe set on the microarray corresponding to each of the S1P receptor, we recorded the number of NPC and normal samples in which a “present” call was made (which provides an indication of expression above an arbitrary threshold). We also performed a statistical test to determine if the level of expression for each probe set was significantly different between NPC samples and normal controls. Data from probe sets that yielded “absent” calls in all samples were considered not valid. Significant change was defined as fold change > 1.5 with a p value < 0.05 . Only S1PR3 was significantly up-regulated in both microarray datasets. NA, not available.