ABERRANT ACTIVATION OF SPHINGOSINE-1-PHOSPHATE SIGNALLING IN NASOPHARYNGEAL CARCINOMA

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a highly metastatic disease arising from the epithelial cells in the nasopharynx that is exceptionally prevalent in Southeast Asia and Southern China. NPC is classified into keratinising and non-keratinising carcinoma in which non-keratinising NPC is consistently associated with Epstein-Barr virus (EBV) infection; close to 100% of cases in endemic regions are EBV-associated. More than 70% of NPC patients present with late stage disease and existing treatment for advanced disease is limited to concurrent chemo-radiotherapy. Approximately 30% of these patients develop distant metastases post therapy and due to the location of tumours in close proximity to many vital organs in the head and neck region, most NPC survivors have an impaired health-related quality of life. A better understanding of the molecular basis of NPC is required to inform innovations in the therapeutic approach. The present study was designed to investigate the biological significance of sphingosine-1-phosphate (S1P) signalling in the pathogenesis of NPC and the contribution of EBV to the dysregulation of this pathway. S1P is a bioactive lipid produced by the activity of sphingosine kinases (SPHKs), which signals through a family of five G protein-coupled receptors, termed S1P receptors 1-5 (S1PR1-5), to trigger multiple pathways that regulate important biological processes. There is now compelling evidence to show that the SPHKs/S1P/S1PRs axis is a novel and attractive therapeutic target in cancer. High expression of SPHK1 has been shown in primary NPCs and therefore, elevated levels of S1P are likely to be present in NPC cells. The present study showed that treatment of NPC cells with exogenous S1P enhanced the migration and invasion and these effects were accompanied by the activation of AKT. Focusing on the migratory phenotype, shRNA knockdown of SPHK1 resulted in a reduction in the levels of phosphorylated AKT and inhibition of cell migration. Furthermore, re-analysis of two published microarray datasets revealed the over-expression of S1PR3 in primary NPC tissues

compared to non-malignant nasopharyngeal epithelium. Knockdown of S1PR3 inhibited the activation of AKT and the S1P-induced migration of NPC cells. The expression of constitutively active AKT was able to partially rescue the repressive effects of the knockdown of SPHK1 and S1PR3 on cell migration. In addition, the only EBV-positive NPC cell line, C666-1, expressed the highest levels of SPHK1 and S1PR3 compared to a panel of seven EBV-negative NPC cell lines. To elucidate the contribution of EBV to the deregulation of S1P signalling, the present study demonstrated that EBV infection or ectopic expression of EBV-encoded latent genes (EBNA1, LMP1 or LMP2A) can upregulate the expression of SPHK1 in NPC cells. Taken together, the results of the present study show for the first time that S1P induces NPC cell migration by activating AKT through S1PR3, and point to a central role of EBV infection in mediating the oncogenic effects of S1P in this disease. Therefore, targeting S1P signalling could be a promising therapeutic intervention for NPC.

ABSTRAK

Kanser nasofarinks (NPC) adalah satu penyakit kanser metastatik yang berkembang daripada sel-sel epitelium dalam nasofarinks dan berleluasa di Asia Tenggara dan China Selatan. NPC dikelaskan kepada karsinoma berkeratin dan tanpa keratin. NPC tanpa keratin dikaitkan pula dengan jangkitan virus Epstein-Barr (EBV) secara konsisten di mana hampir 100% kes-kes NPC di kawasan-kawasan endemik berhubungkait dengan EBV. Lebih daripada 70% pesakit NPC hanya dapat dikesan pada tahap yang lewat dan rawatan bagi penyakit peringkat lewat adalah terhad kepada kombinasi kemoterapi dan radioterapi. Ketumbuhan dalam lebih kurang 30% pesakit di tahap lewat merebak ke bahagian-bahagian badan yang lain selepas terapi dan disebabkan oleh lokasi ketumbuhan yang berdekatan dengan banyak organ penting di bahagian kepala dan leher, kebanyakan pesakit kanser yang terselamat mempunyai kualiti hidup yang terjejas. Untuk menghasilkan inovasi dalam pendekatan terapeutik, pemahaman yang lebih mendalam tentang asas molekul NPC amat diperlukan. Kajian ini bertujuan untuk menyiasat kepentingan biologi isyarat sphingosine-1-fosfat (S1P) dalam patogenesis NPC dan sumbangan EBV kepada penyahkawalseliaan jalur isyarat ini. S1P adalah satu molekul lipid bioaktif yang dihasilkan oleh enzim-enzim sphingosine kinases (SPHKs) dan menghasilkan isyarat melalui famili yang mengandungi lima jenis G protein reseptor ditambah, bernama reseptor S1P 1-5 (S1PR1-5). Penghasilan isyarat ini mengaktifkan pelbagai jalur yang mengawal proses penting dalam sistem biologi manusia. Kini terdapat bukti kukuh yang menunjukkan bahawa paksi SPHKs/S1P/S1PRs adalah sasaran terapeutik yang novel dan menarik dalam kanser. Ekspresi tinggi SPHK1 telah ditunjukkan dalam tisu NPC. Oleh itu, peningkatan tahap S1P mungkin berlaku dalam sel-sel NPC. Kajian ini menunjukkan bahawa rawatan sel-sel NPC dengan S1P meningkatkan migrasi dan invasi dalam sel-sel tersebut dan kesan-kesan ini ditemani oleh pengaktifan AKT. Dengan memberi tumpuan kepada fenotip

migrasi, kesan SPHK1 knockdown oleh shRNA telah menyebabkan pengurangan fosforilasi AKT dan perencatan migrasi sel-sel NPC. Tambahan pula, hasil analisis dua set data microarray yang telah diterbitkan menunjukkan peningkatan ekspresi S1PR3 dalam tisu NPC berbanding dengan epitelium nasofarinks yang bukan malignan. S1PR3 knockdown menghalang pengaktifan AKT dan migrasi sel-sel NPC yang disebabkan oleh S1P. Ekspresi AKT yang aktif secara konstitutif dapat memulihkan sebahagian kesan perencatan migrasi sel yang berpunca daripada SPHK1 dan S1PR3 knockdown. Di samping itu, C666-1 yang merupakan satu barisan sel NPC yang EBV-positif sahaja, menunjukkan ekspresi SPHK1 dan S1PR3 yang tertinggi berbanding dengan tujuh barisan sel NPC lain yang EBV-negatif. Untuk menjelaskan sumbangan EBV dalam deregulasi jalur S1P, kajian ini telah menunjukkan bahawa jangkitan EBV atau ekspresi ektopik gen laten EBV (EBNA1, LMP1 atau LMP2A) dapat meningkatkan ekspresi SPHK1 dalam sel-sel NPC. Oleh itu, buat pertama kalinya, kajian ini menunjukkan bahwa S1P menyebabkan sel-sel NPC bermigrasi dengan mengaktifkan AKT melalui S1PR3, dan mengetengahkan peranan penting jangkitan EBV sebagai pengantara kesan onkogenik S1P dalam penyakit ini. Justeru, penyasaran jalur S1P menunjukkan potensi dalam intervensi secara terapeutik bagi menangani penyakit NPC.

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AKT	:	Protein kinase B
AP-1	:	Activator protein 1
BACE1	:	β-amyloid precursor protein cleaving enzyme 1
BARF1	:	*Bam*HI-A reading frame-1
BARTs	:	*Bam*HI-A rightward transcripts
BL	:	Burkitt's lymphoma
BSA	:	Bovine serum albumin
CR2	:	Complement receptor 2
CSF1R	:	Colony stimulating factor 1 receptor
CTAR1	:	C-terminal activation region 1
CTAR2	:	C-terminal activation region 2
dsRNA	:	Double-stranded RNA
EBER	:	EBV-encoded RNA
EBNA	:	Epstein-Barr virus nuclear antigen
EBV	:	Epstein-Barr virus
ECL	:	enhanced chemiluminescene
EGF	:	Epidermal growth factor
EMT	:	Epithelial-mesenchymal transition
ERK	:	Extracellular signal-regulated kinase
FAFA	:	Fatty acid-free albumin
FBS	:	Fetal bovine serum
GPCR	:	G protein-coupled receptor
GWAS	:	Genome-wide association studies
HDAC	:	Histone deacetylases

HIF-1α	:	Hypoxia-inducible factor-1α
HINGS	:	Heat-inactivated normal goat serum
HLA	:	Human leukocyte antigen
HNSCC	:	Head and neck squamous cell carcinoma
HRP	:	horseradish peroxidase
hTERT	:	human telomerase reverse transcriptase
IFN	:	Interferon
Ig	:	Immunoglobulin
IGF	:	Insulin-like growth factor
IL	:	Interleukin
ITAM	:	Immunoreceptor tyrosine-based activation motif
JNK	:	c-Jun N-terminal kinase
kb	:	Kilobase pair
LCL	:	Lymphoblastoid cell line
LMP	:	Latent membrane protein
LPA	:	Lysophosphatidic acid
MAPK	:	Mitogen-activated protein kinase
miRNA	:	MicroRNA
mRNA	:	Messenger RNA
NF- κB	:	Nuclear factor-kappa B
NK	:	Natural killer
NOD/SCID	:	Non-obese diabetic/severe combined immunodeficiency
NPC	:	Nasopharyngeal carcinoma
PDX	:	Patient-derived xenograft
PI3K	:	Phosphatidylinositol-3-kinase
PKR	:	Protein kinase R

PML	:	Promyelocytic leukaemia
PPARγ	:	Peroxisome proliferator-activated receptor γ
PVDF	:	Polyvinylidene difluoride
Q-PCR	:	Quantitative polymerase chain reaction
RIG-1	:	Retinoic acid–inducible gene 1
RNA	:	Ribonucleic acid
S1P	:	Sphingosine-1-phosphate
S1PR	:	Sphingosine-1-phosphate receptor
SDS-PAGE	:	SDS-polyacrylamide gel electrophoresis
shRNA	:	Short hairpin RNA
siRNA	:	Short interfering RNA
SPHK	:	Sphingosine kinase
STAT3	:	Signal transducer and activator of transcription 3
TBS	:	Tris buffered saline
TBST	:	Tris buffered saline tween
TLR3	:	Toll-like receptor 3
TNF	:	Tumour necrosis factor
TRAF2	:	TNF receptor-associated factor 2
VCA	:	Viral capsid antigen
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization
WNT5A	:	Wingless-type MMTV integration site family member 5A
Wp	:	W promoter

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Nasopharyngeal carcinoma (NPC) is a tumour arising in the nasopharynx (Figure 1.1) that is particularly prevalent in Southern China and Southeast Asia (Razak *et al.*, 2010; Torre *et al.*, 2015). Due to the unspecific symptoms of NPC, more than 70% patients are diagnosed at advanced stages (Razak *et al.*, 2010) and approximately 30% of these patients develop distant metastases following initial treatment (Tao *et al.*, 2007; Wu *et al.*, 2016). The current mainstay of treatment for advanced NPC is concurrent chemoradiotherapy (Chua *et al.*, 2015). Unfortunately, due to the close proximity of the tumours to many vital organs in the head and neck region, most NPC patients suffer from poor quality of life after the treatment (Du *et al.*, 2015). Despite a developing understanding of the molecular basis of NPC, currently there no biomarkers or targeted therapies available in the clinic. Therefore, novel therapeutic strategies are urgently needed for a better management of NPC patients.

NPC is divided into two histopathological types, namely keratinising squamous cell carcinoma and non-keratinising carcinoma. Non-keratinising NPC constitutes most, if not all, of the NPC cases in endemic regions and is consistently associated with Epstein-Barr virus (EBV) infection (Young *et al.*, 2014). The expression of EBV latent genes in NPC is restricted to Epstein-Barr nuclear antigen 1 (EBNA1), latent membrane proteins (LMP1 and LMP2), EBV-encoded RNAs (EBERs), *Bam*HI-A rightward transcripts (BARTs) and *Bam*HI-A Reading Frame-1 (BARF1). Although the exact pathogenic role of EBV in NPC remains enigmatic, EBV-encoded genes have been shown to alter a number of important cellular processes in nasopharyngeal epithelial cells, which contributes to the development of NPC (Tsao *et al.*, 2015; Young *et al.*, 2014).

Given that distant metastasis is the major cause of death in NPC patients, this study aimed to investigate the functional role of signalling pathways that contribute to the migratory and invasive properties of NPC cells, with a focus on sphingosine-1-phosphate (S1P) signalling. S1P is a bioactive lipid produced by the phosphorylation of sphingosine by sphingosine kinases 1 and 2 (SPHK1 and SPHK2). Following binding of S1P to a family of G-protein coupled receptors (termed S1PR1 – S1PR5), diverse downstream signalling pathways are activated, which subsequently regulate a number of cellular processes in normal physiology, such as lymphocyte trafficking and vascular integrity (Spiegel *et al.*, 2011). Accumulating evidence has shown that aberrant S1P signalling contributes to tumorigenesis and has identified an oncogenic role for SPHK1 (Pyne *et al.*, 2010). High expression of SPHK1 is associated with reduced patient survival in various types of cancer, including NPC (Li *et al.*, 2015b; Pyne *et al.*, 2016). High expression of SPHK1 in tumours results in elevated levels of S1P and this, in turn, promotes tumorigenesis by increasing cell migration, invasion, proliferation, survival and angiogenesis (Pitson, 2011).

The oncogenic effects of S1P can occur as a result of alterations in the expression or function of the S1P receptors (Blaho *et al.*, 2014). For example, S1PR1 and S1PR3 have been shown to promote cancer development, whereas S1PR2 is generally thought to inhibit migration, invasion and metastasis, although recent evidence suggests that S1PR2 can also have tumour-promoting effects by increasing cancer cell growth and migration (Adada *et al.*, 2013; Beckham *et al.*, 2013; Patmanathan *et al.*, 2016; Takuwa *et al.*, 2011). The roles of S1PR4 and S1PR5 in cancer remain largely unclear (Adada *et al.*, 2013). Since aberrant activation of the S1P signalling pathway has been implicated in various malignancies, targeting the SPHK1/S1P/S1PR axis has emerged as a promising new strategy to treat cancer. Several drugs targeting this pathway have

undergone clinical trials in cancer and the new drugs with higher specificity and efficacy are being developed (Kunkel *et al.*, 2013).

High expression of SPHK1 in NPC has been reported previously (Li *et al.*, 2015b), but the downstream biological consequences of elevated SPHK1 in NPC cells have not been studied. The aims of the present study were to investigate the functional consequences of aberrant activation of the SPHK1/S1P/S1PR axis in NPC and to examine the contribution of EBV infection to the deregulation of S1P signalling in this disease.

1.2 General Aims

EBV-associated NPC is a highly metastatic disease with poor patient prognosis (Khan *et al.*, 2014) and thus there is a compelling need to identify novel therapeutic targets that can improve the management of NPC patients. Although the contribution of aberrant S1P signalling to tumorigenesis has been convincingly shown in various types of cancer, its involvement in NPC remains to be elucidated. Therefore, this study was initiated to investigate the role of S1P signalling in the pathogenesis of NPC and to determine whether EBV infection leads to the dysregulation of this pathway.

Elevated levels of S1P are likely to be present in NPC as a consequence of high expression of SPHK1. Therefore, the first part of this study investigated whether exogenous S1P would affect the phenotypic characteristics (proliferation, migration and invasion) of NPC cells and whether these effects could be reversed by the knockdown of SPHK1. Focusing on the migratory phenotype, subsequent experiments aimed to identify which of the two well-known downstream targets of S1P [protein kinase B (AKT) and extracellular signal-regulated kinase (ERK)] might be involved.

Having confirmed S1P induced NPC cell migration through the activation of AKT, the second part of this study explored which S1P receptors might be responsible for these effects. Re-analysis of previous two microarray datasets identified S1PR2 and S1PR3 as candidate receptors based on their significantly higher expression in primary NPC tissues compared to non-malignant nasopharyngeal epithelium. Treatment of NPC cells with pharmacological drugs specific for these two receptors revealed the potential involvement of S1PR3. Knockdown experiments targeting S1PR3 were subsequently performed to confirm its role in S1P-induced migration and AKT activation.

Given that all non-keratinising NPC cells carry EBV genomes, the final part of this study investigated whether EBV infection contributed to the aberrant activation of S1P signalling in NPC cells. Two NPC cell lines that expressed low levels of SPHK1 and S1PR3 were stably infected with a recombinant EBV (Akata strain). During the course of this study, a number of established EBV-infected nasopharyngeal epithelial cell lines were also obtained from collaborators. Using these cell models, the contribution of EBV infection and EBV-encoded latent genes to the expression of SPHK1 and S1PR3 was examined.

1.3 Objectives

The objectives of this study were as follows:

(i) To determine the biological significance of exogenous sphingosine-1-phosphate (S1P) and knockdown of sphingosine kinase 1 (SPHK1) on the behaviour of NPC cells *in vitro*

(ii) To identify the S1P receptor(s) that is/are responsible for S1P-mediated migration in NPC

(iii) To investigate the contribution of Epstein-Barr virus (EBV) infection to the expression of SPHK1 and S1P receptor 3 (S1PR3) in NPC cells

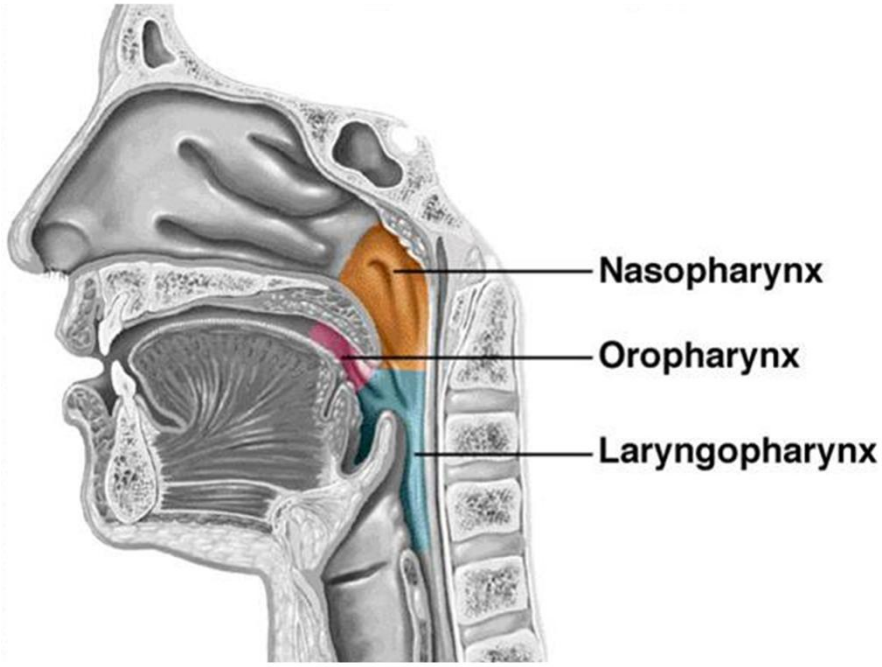


Figure 1.1: Regions of pharynx

The location of the nasopharynx (brown), oropharynx (pink) and laryngopharynx (blue) is shown.

Figure adapted from http://fau.pearlashes.com/anatomy.

CHAPTER 2: LITERATURE REVIEW

2.1 The biology of cancer

Cancer is one of the leading causes of mortality worldwide (Mortality *et al.,* 2015). Globally, it was estimated that an overall of 14.1 million of new cancer cases and 8.2 million of cancer-related deaths occurred in 2012 (Torre *et al.*, 2015). The development of cancer is a complex, multi-step process that ultimately leads to uncontrolled cell growth. Molecular alterations in oncogenes and tumour suppressor genes are well-recognised as the major factors contributing to the malignant phenotype (Cairns *et al.*, 2011).

In 2000, Hanahan and Weinberg proposed that all cancers share six common traits ("hallmarks"); cancer cells are self-sufficient in growth signals, insensitive to anti-growth signals, able to evade apoptosis, have limitless replicative potential, can sustain angiogenesis and possess invasive and metastatic potentials (Hanahan *et al.*, 2000). In 2011, these authors proposed two additional hallmarks of cancer, namely reprogramming of cellular energy metabolism and evasion of immune destruction (Hanahan *et al.*, 2011). The acquisition of these eight hallmarks of cancer was suggested to be facilitated by two enabling characteristics, which are genomic instability and mutation, and tumour-promoting inflammation (Hanahan *et al.*, 2011).

2.2 Nasopharyngeal carcinoma

NPC is a malignancy arising from the epithelial cells in the nasopharynx. NPC is rare in most parts of the world with an incidence rate of less than 1 per 100,000 persons per year, but it is particularly prevalent in regions such as Southern China and Southeast Asia (Parkin *et al.*, 2005). NPC is also found in Eskimos from Greenland and Alaska, and populations within North Africa (Parkin, 2006). The incidence rate of NPC peaks at the age of 50 to 59 years and is 2- to 3-fold higher in males compared to females

(Chang *et al.*, 2006; Torre *et al.*, 2015). In Malaysia, 940 new cases of NPC were diagnosed in 2007 with 685 cases in males (73%) and 255 cases in females (27%). Overall, NPC represented the fourth most common cancer and the third leading cancer among males in Malaysia (Omar, 2007).

2.2.1 Histopathology

In 1991, the WHO classified NPC into two histopathological types, namely keratinising squamous cell carcinoma and non-keratinising carcinoma, in which the latter is further subdivided into differentiated and undifferentiated carcinoma (Shanmugaratnam *et al.*, 1991). Keratinising NPC is characterised by well-differentiated histological features including the presence of intercellular bridges, keratin production and epithelial pearl formation. In contrast, non-keratinising NPC lacks keratinisation features and sheets of epithelial cells show syncytial architecture with lymphocytes intimately associated with the neoplastic cells. Undifferentiated NPC displays a prominent lymphocytic component and it is also referred as "lymphoepithelioma". Keratinising NPC is usually seen in low incidence areas while non-keratinising NPC accounts for majority of the NPC cases in endemic regions (Lo *et al.*, 2004b; Marcus *et al.*, 2010).

2.2.2 Aetiology

Epidemiological studies suggest three major aetiological factors for NPC, namely genetic susceptibility, environmental factors and EBV infection (Lo *et al.*, 2004b). These aetiological factors may contribute independently or jointly to the development of NPC (Chang *et al.*, 2006).

2.2.2.1 Genetic susceptibility

The observation that second and third generation Chinese emigrants from endemic regions to low incidence areas have a higher risk of developing NPC than Caucasians

suggested that genetic susceptibility plays a critical role in the development of NPC (Buell, 1974). Early linkage studies on Chinese sib pairs with NPC revealed a NPC genetic susceptibility locus within the human leukocyte antigen (HLA) region (Lu *et al.*, 1990). The HLA genes encode proteins required for the identification and presentation of foreign antigens, including EBV-encoded peptides, to trigger host immune responses. Increased risk of NPC has been found to be associated with HLA alleles A2, B14 and B46, whilst alleles A11, B13 and B22 were found to have protective effects (Goldsmith *et al.*, 2002). With the advances in genotyping technologies, a number of genome-wide association studies (GWAS) have also consistently revealed the association of NPC with HLA genes on chromosome 6p21 (*HLA-A*, *HLA-B*, *HLA-C*, *HLA-DR*, *HLA-DQ* and *HLA-F*) (Bei *et al.*, 2010; Tang *et al.*, 2012; Tse *et al.*, 2009; Zhao *et al.*, 2012). Several non-HLA genes also located within the HLA region, including *GABBR1*, *HCG9*, *MICA* and *HCP5*, were also found to be associated with NPC (Tse *et al.*, 2009; Tse *et al.*, 2011). In addition, other genetic susceptibility loci for NPC identified from GWAS studies include *TNFRSF19* (13q12), *MDS1-EVI1* (3q26), *CDKN2A* (9p21), *CDKN2B* (9p21), *ITGA9* (3p21) and *MST1R* (3p21) (Bei *et al.*, 2010; Dai *et al.*, 2016; Ng *et al.*, 2009).

Other potential susceptibility genes that have been shown to be associated with an increased risk of developing NPC include genes responsible for nitrosamine metabolism (*CYP2E1*, *CYP2A6*), detoxification of carcinogens (*GSTM1*), DNA repair (*hOGG1*, *XRCC1*), interleukins (*IL1α*, *IL10*, *IL16*, *IL18*) and telomere maintenance (*TERT/CLPTM1L)* (Bei *et al.*, 2016; Cheng *et al.*, 2014; Cho *et al.*, 2003; Guo *et al.*, 2013; Hildesheim *et al.*, 1997; Nazar-Stewart *et al.*, 1999; Qin *et al.*, 2014; Tiwawech *et al.*, 2006; Tsai *et al.*, 2014; Yee Ko *et al.*, 2014). A systemic review of 83 published papers confirmed the correlation of increased NPC susceptibility with HLA genes and also identified several genes involved in DNA repair (*RAD51L1*), cell-cycle checkpoint

regulation (*MDM2*, *TP53*), cell adhesion and migration (*MMP2*) (Hildesheim *et al.*, 2012).

2.2.2.2 Environmental factors

Dietary habits are also thought to influence the risk of developing NPC. Consumption of salted fish, particularly during childhood, has been strongly associated with an increased NPC risk (Armstrong *et al.*, 1983; Guo *et al.*, 2009; Ning *et al.*, 1990; Yu *et al.*, 1986). This risk is also elevated with the intake of other preserved food such as fermented bean paste and preserved vegetables (Yu *et al.*, 1989; Yu *et al.*, 1988). The presence of carcinogenic volatile nitrosamines in preserved foods is believed to be the main contributing factors (Poirier *et al.*, 1989; Yu *et al.*, 1988; Zou *et al.*, 1994). Usage of traditional herbal medicines has also been suggested to be a risk factor of NPC among Asian populations by stimulating the expression of EBV lytic antigens in the host (Furukawa *et al.*, 1986; Hildesheim *et al.*, 1992). In contrast, consumption of fresh fruits and/or vegetables, especially during childhood, is considered to have a protective effect (Chang *et al.*, 2006; Yu *et al.*, 1989).

Some non-dietary factors have also been found to contribute to the risk of developing NPC and these include occupational exposure to toxic pollutants (formaldehyde) in the air, wood dust, and textiles, which possibly induce chronic irritation and inflammation in the nasopharynx (Armstrong *et al.*, 1983; Chang *et al.*, 2006; Li *et al.*, 2006b; Sriamporn *et al.*, 1992). While long term cigarette smoking has been associated with increased incidences of keratinising NPC in low-risk populations (Cheng *et al.*, 1999; Vaughan *et al.*, 1996; Zhu *et al.*, 2002), its association with non-keratinising NPC in endemic areas remains controversial (Chen *et al.*, 1990; Lanier *et al.*, 1980; Sriamporn *et al.*, 1992; Zou *et al.*, 2000).

2.2.2.3 EBV infection

Non-keratinising NPC is consistently associated with EBV infection (Niedobitek, 2000). The association of NPC and EBV infection was initially suggested when Burkitt lymphoma antigen-specific antibodies were also detected in the serum of NPC patients (Old *et al.,* 1966). Subsequent serological analyses showed a correlation between EBV antibody titres and NPC tumour stage, and identified viral capsid antigen (VCA)-specific IgA as a prognostic marker (Henle *et al.*, 1976; Zeng *et al.*, 1982; Zong *et al.*, 1992).

EBV establishes latent infection in NPC cells and the viral genome is maintained episomally (Niedobitek *et al.*, 1996). The observation that EBV genomes were detected in pre-invasive dysplastic lesions or carcinoma *in situ* of the nasopharynx suggested that EBV infection might be an early event in the development of NPC (Pathmanathan *et al.*, 1995). The contribution of EBV infection and EBV-encoded genes to the pathogenesis of NPC is further discussed in Section 2.3.

2.2.3 Clinical presentation, diagnosis and treatment

The early clinical symptoms of NPC are usually unspecific, for example epistaxis, nasal obstruction and auditory complaints (Tabuchi *et al.*, 2011) and this results in late presentation; the majority of the NPC cases are diagnosed at advanced stages (Razak *et al.*, 2010). NPC is a highly metastatic disease with neck lumps being found in approximately three-quarters of patients and distant metastasis remains the major cause of death in NPC patients (Chua *et al.*, 2015). In many cases, distant metastasis appears within 18 months after the presenting symptoms (Cvitkovic *et al.*, 1993) and the median survival time of these patients is only approximately 9 – 12 months (Tao *et al.*, 2007).

Currently, the diagnosis of NPC depends on the pathological examination of biopsy specimens (Jeyakumar *et al.*, 2006). Staging of NPC is determined according to the

tumour, node, metastasis (TNM) classification of the American Joint Committee on Cancer (Barnes *et al.*, 2005). Imaging modalities such as computed tomography (CT) scans and magnetic resonance of imaging (MRI) are widely used to assess tumour extension and disease stage (Brennan, 2006). Technological advances in recent years have allowed the invention of ^{18}F-2-fluoro-2-deoxy-d-glucose (FDG) positron emission tomography-computed tomography (PET/CT) that shows considerable promise in the diagnosis, therapy assessment and prognosis of the disease (Agarwal *et al.*, 2013). Other molecular-based methods, such as EBV serological tests and quantitative analysis of EBV DNA have also been proposed as non-invasive and economic diagnostic tests for NPC and may be useful in the near future (Tao *et al.*, 2007).

The primary treatment for early stage NPC (stage I and IIa) is normally radiotherapy alone. With the advent of modern radiation technology, intensity-modulated radiotherapy (IMRT), which can provide tumourcidal doses to the tumour while minimising doses to the adjacent normal tissues, is currently the preferred treatment for NPC over standard 2D conventional radiotherapy (Xu *et al.*, 2013). Concurrent chemoradiotherapy with or without adjuvant chemotherapy is the mainstay of treatment for locoregionally advanced diseases (Chua *et al.*, 2015). The presence of EBV in all NPC cells has also provided opportunities for the development of novel therapeutic interventions such as EBV-based immunotherapies that may lead to a better management of NPC patients in the future (Tsang *et al.*, 2014).

2.2.4 Molecular basis of NPC

Cytogenetic studies have revealed multiple chromosomal abnormalities in NPC; consistent genetic losses have been identified on chromosome 3p, 9p, 9q, 11q, 13q, 14q and 16q, while chromosomal gains occur on chromosome 1q, 3q, 7q, 8q, 11q, 12p, 12q, 19p and 19q (Fang *et al.*, 2001; Hui *et al.*, 1999; Li *et al.*, 2006c; Wong *et al.*, 2003).

Loss of heterozygosity (LOH) on chromosome 3p and 9p occurs is thought to be an early event in the progression of NPC (Chan *et al.*, 2002; Chan *et al.*, 2000).

The identification of the chromosomal loci that frequently harbour gross structural abnormalities informed studies that identified specific genes might be involved in the development of NPC. Deletion or promoter hypermethylation of *RASSF1A* on chromosome 3p and *p16* (*CDKN2A*) on chromosome 9p are recognised as early events in NPC tumorigenesis (Kwong *et al.*, 2002; Lo *et al.*, 1996; Young *et al.*, 2004). Other tumour suppressor genes on chromosome 3p (*BLU/ZMYND10*, *DLEC1*, *PTPRG* and *FBLN2*) (Cheung *et al.*, 2008; Kwong *et al.*, 2007; Law *et al.*, 2012; Liu *et al.*, 2003) and chromosome 11q (*TSLC1*, *THY1*, *CRYAB*) have also been identified (Hui *et al.*, 2003; Lung *et al.*, 2005; Lung *et al.*, 2008). Moreover, several oncogenes include *BCL-2*, *LTBR*, *CCDN1*, *PIK3CA*, *C-MYC*, *RAS* and *Bmi-1* have been shown to be amplified or exhibit gain-of-function mutations (Hui *et al.*, 2005; Lo *et al.*, 2012; Lu *et al.*, 1993; Or *et al.*, 2010; Or *et al.*, 2006; Porter *et al.*, 1994). Over-expression of some of the oncogenes such as *LTBR* and *PIK3CA* in pre-cancerous lesions or NPC tumours has been reported to be critical in the pathogenesis of NPC through activation of multiple signalling pathways, including nuclear factor-kappa B (NF-κB) and phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) (Lo *et al.*, 2012). Recent whole exome sequencing (WES) studies of NPC have revealed particular genetic alterations, such as deletions and/or mutations of multiple genes involved in chromatin modification (*ARID1A*, *BAP1*), autophagy machinery (*ATG2A*, *ATG7*, *ATG13*), ERBB-PI3K signalling pathway (*PIK3CA*, *ERBB2*, *ERBB3*), NF-κB signalling pathway (*NFKBIA*, *CYLD*, *TNFAIP3*) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-mediated signatures (*APOBEC3A*, *APOBEC3B*), that may contribute to the development of NPC (Lin *et al.*, 2014a; Zheng *et al.*, 2016). It is of interest that although earlier studies had reported infrequent *TP53* mutations in NPC

(Spruck *et al.*, 1992), these powerful WES approaches reveal *TP53* is the most frequently mutated gene in NPC (7-10%), albeit the frequency is still much lower compared to other human cancers (Petitjean *et al.*, 2007).

The role of EBV in the pathogenesis of NPC is thought to result from the aberrant establishment of virus latent infection in epithelial cells displaying pre-malignant changes, such as overexpression of cyclin D1 and/or *p16* deletion (Tsang *et al.*, 2012). Secretion of inflammatory cytokines by EBV-infected NPC cells has been suggested to support EBV latent infection and malignant transformation of the infected cells (Huang *et al.*, 1999). In particular, interleukin (IL) 6 has been shown to support the persistence of EBV latent infection in infected NPC cells. IL6 activates signal transducer and activator of transcription 3 (STAT3) signalling that in turn regulates the transcription of EBNA1 which governs the maintenance of the EBV episome in infected cells (Chen *et al.*, 2003). A positive feedback loop to support EBV latent infection was also established between LMP1 and STAT3 in which LMP1 induces IL6 secretion to activate STAT3 and that in turn upregulates the expression LMP1 (Chen *et al.*, 2003). EBV latent genes also deregulates a number of signalling pathways, promotes genetic instability, stimulates epigenetic changes, modulates tumour microenvironment and suppresses host immune response to provide growth and survival benefits to the NPC cells (Lo *et al.*, 2012; Tsao *et al.*, 2015). A possible model of NPC pathogenesis has been proposed and depicted in Figure 2.1.

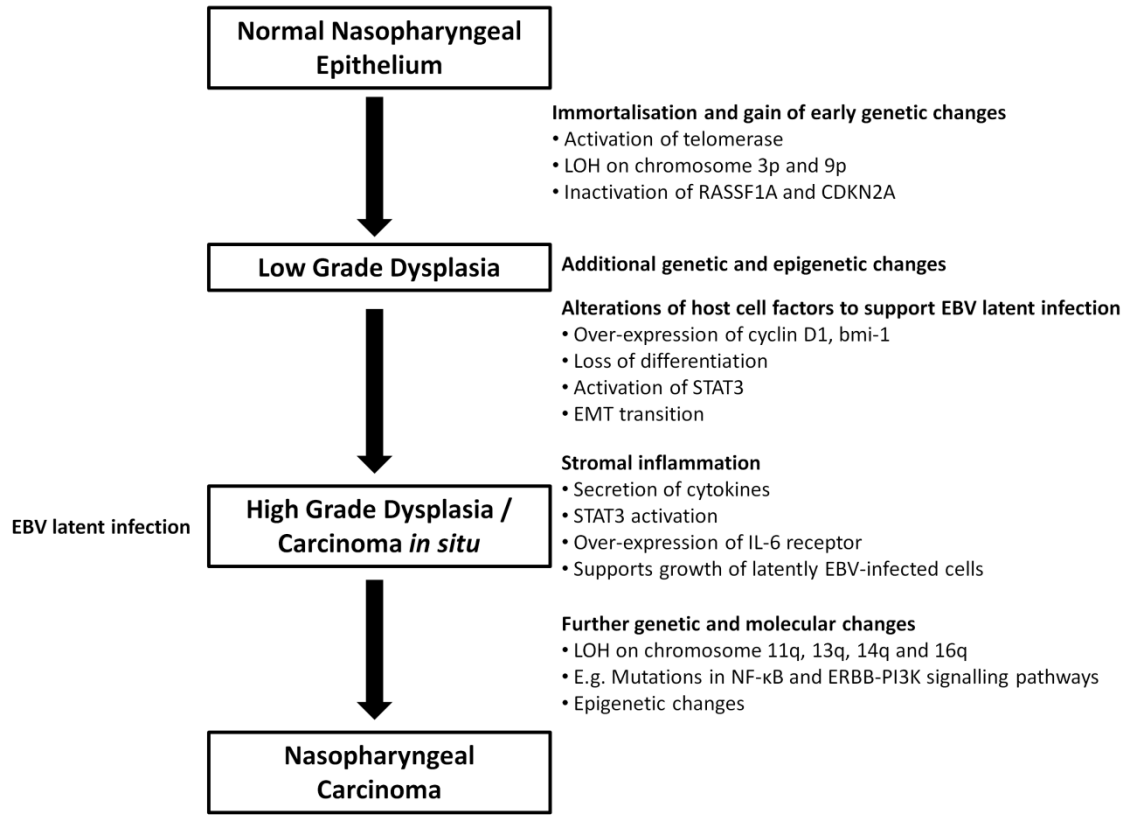


Figure 2.1: Model of NPC pathogenesis

A possible model of NPC pathogenesis. Activation of telomerase, loss of heterozygosity (LOH) on chromosome 3p and 9p, and inactivation of RASSF1A and CDKN2A occur early in the pathogenesis of NPC to promote the formation of low grade dysplasia. The accumulation of additional genetic and epigenetic changes may facilitate and support EBV latent infection. Acting together with stromal inflammation, further genetic and molecular alterations (for example mutations in NF-κB and ERBB-PI3K signalling pathways) in the nasopharyngeal epithelial cells ultimately lead to the development of NPC.

Figure modified from Tsao *et al.*, 2014.

2.3 Epstein-Barr virus

EBV is a γ-herpesvirus that was discovered in 1964 from a Burkitt's lymphoma (BL) biopsy (Epstein *et al.*, 1964). EBV infects more than 90% of human population through bodily fluids, primarily saliva (Odumade *et al.*, 2011). Once the host is infected, the infection remains lifelong (Henle *et al.*, 1979). Primary infection with EBV usually occurs during early childhood and it is asymptomatic in most cases, particularly in developing countries (Biggar *et al.*, 1978; Jenson, 2000). However, in most developed countries, primary infection is delayed into late adolescence or adulthood and this often results in a self-limiting lymphoproliferative disease called infectious mononucleosis (Henle *et al.*, 1968; Niedobitek *et al.*, 2001).

The oncogenic potential of EBV was initially identified by its ability to transform normal resting B lymphocytes into permanently growing lymphoblastoid cell lines (LCLs) (Henle *et al.*, 1967; Pope *et al.*, 1968). In addition to BL, EBV infection was subsequently found to be associated with a number of malignancies of both lymphoid and epithelial origin, including Hodgkin's lymphoma, extranodal natural killer (ENK)/T-cell lymphoma, NPC and gastric carcinoma (Murray *et al.*, 2001). EBV infection is common in immunocompromised individuals, resulting in lymphoproliferative diseases, such as X-linked lymphoproliferative disease, post-transplant lymphoproliferative disorder and AIDS-related lymphoproliferative disorder (Thompson *et al.*, 2004).

2.3.1 EBV genome and sequence variation

The EBV genome is composed of linear double-stranded DNA, approximately 172 kilobase pairs (kb) in length that encodes more than 85 genes. EBV (strain B95-8) was the first human herpesvirus to have its genome fully cloned and sequenced (Baer *et al.*, 1984). The EBV genome consists of a series of 0.5 kb terminal repeats at each terminus

and approximately 3 kb internal repeat sequences that divide the viral genome into unique, short and long regions (Baer *et al.*, 1984; Cheung *et al.*, 1982). When EBV infects a cell, the viral genome forms a circular episome through covalent fusion of the terminal repeat sequences (Figure 2.2) (Raab-Traub *et al.*, 1986).

There are two types of EBV, type 1 (EBV-1) and type 2 (EBV-2), which differ mainly in the sequences of EBNA2 and EBNA3 genes (Rowe *et al.*, 1989; Sample *et al.*, 1989). EBV-1 strains are more prevalent worldwide (Zimber *et al.*, 1986) and have been shown to transform B cells more efficiently than EBV-2 *in vitro* (Rickinson *et al.*, 1987). This might be attributable to the greater ability of EBV-1 strains to maintain the growth of infected cells through the EBNA2-mediated expression of CXCR7 and LMP1 (Cancian *et al.*, 2011; Lucchesi *et al.*, 2008; Tzellos *et al.*, 2014).

It has been hypothesized that EBV strain variations might account for the different incidence rates of EBV-associated diseases in different parts of the world, but this has so far not been conclusively proven. Eighteen years after the first complete sequence of EBV strain B95-8 was published (Baer *et al.*, 1984), a "wild type" EBV sequence (EBVwt) was constructed using B95-8 as a backbone, while a 12-kb deleted segment (encoding some of the BART miRNA genes and one of the origins of lytic replication) was provided by the sequences from Raji strain (de Jesus *et al.*, 2003). Finally, the current reference sequence of EBV that included three additional small open reading frames was released in 2010 as the RefSeq HHV4 (EBV) sequence (GenBank accession number NC_007605). Subsequently, complete sequences of two EBV strains (GD1 and AG876) were published using similar Sanger sequencing methods (Dolan *et al.*, 2006; Zeng *et al.*, 2005). With the advances in next generation sequencing (NGS) technology, the genome sequences of 18 additional EBV strains have been reported since 2011; eleven strains from NPC (GD2, M81 and HKNPC1 to HKNPC9) (Kwok *et al.*, 2012;

Kwok *et al.*, 2014; Liu *et al.*, 2011; Tsai *et al.*, 2014), two strains from BL (Akata and Mutu) (Lin *et al.*, 2013), and five strains from immortalized B-lymphocyte cultures (K4123-Mi, K4413-Mi and three genomes from the 1000 Genome project) (Lei *et al.*, 2013a; Santpere *et al.*, 2014). More recently, complete sequences of 71 geographically distinct EBV strains were published, representing the most comprehensive analysis to date (Palser *et al.*, 2015). It has now become clear that while there is a high level of overall similarity among the virus strains, variations exist in some viral genes that might give rise to functional differences. In particular, the M81 EBV strain isolated from a NPC patient has the tendency to spontaneously switch to lytic replication in B cells but exhibits high propensity to infect epithelial cells (Tsai *et al.*, 2013). In general, NGS analyses have revealed latent genes harbour the highest variation, ranging from single base mutations to extensive insertions and deletions. These findings are in line with early studies showing that the EBV variant with a 30bp deletion in LMP1 has a higher transforming ability by increasing the activation of NF-κB and activator protein 1 (AP-1) and it is associated with a higher risk of distant metastasis in NPC patients (Blake *et al.*, 2001; Hu *et al.*, 1993; Hu *et al.*, 1991; Johnson *et al.*, 1998; Pai *et al.*, 2007). However, the differences in the biological properties of the variants and/or their disease association are yet to be further elucidated.

2.3.2 EBV lytic and latent cycles

EBV displays two distinct lifecycles, namely the latent cycle during persistent infection and the productive lytic phase. The lytic cycle can be activated by diverse stimuli including phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate (TPA) and sodium butyrate (Kenney, 2007). The origin of lytic replication is known as *oriLyt* and the gene responsible for the latent to lytic switch is *BZLF1* (Flemington *et al.*, 1990; Hammerschmidt *et al.*, 1988; Ragoczy *et al.*, 1998). Induction of lytic cycle subsequently stimulates a temporal and ordered cascade of viral gene expression; the

early genes are required for viral DNA replication and nucleotide metabolism and the late genes encode structural proteins for virion packaging (Hislop *et al.*, 2007).

In contrast to lytic replication, latent infection of EBV does not produce progeny virions. In EBV-transformed LCLs, the EBV genome replicates along with the host DNA as an extrachromosomal episome and this process is initiated at the replication origin, *oriP* (Umar, 2006; Yates *et al.*, 1985). During latent infection, a limited set of viral genes named latent genes are expressed, which comprise six nuclear antigens [EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP (leader protein)], three latent membrane proteins (LMP1, LMP2A and LMP2B), EBV-encoded RNAs (EBERs), *Bam*HI-A rightward transcripts (BARTs) and *Bam*HI-H rightward open reading frame 1 (BHRF1) micro-RNAs (miRNAs) (Amoroso *et al.*, 2011; Kang *et al.*, 2015). This pattern of latent EBV gene expression is referred to as the latency III. During different stages of B cell differentiation *in vivo*, alternative forms of EBV latency were identified, namely latency II [expression of EBNA1, LMP1, LMP2, EBERs, BARTs and *Bam*HI-A open reading frame 1 (BARF1)] or latency I (only EBNA1, EBERs and BARTs are expressed) (Thompson *et al.*, 2004; Young *et al.*, 2014). EBV-associated B cell lymphomas express either latency I, II or III, whilst EBV-associated epithelial cancers express a latency II programme (Table 2.1) (Young *et al.*, 2004). Deviations in the pattern of EBV gene expression from these classifications have also been observed. For example, a subset of BL tumours expresses additional viral genes including EBNA3A, EBNA3B, EBNA3C and EBNA-LP, together with an EBNA2 deletion (Kelly *et al.*, 2002). This is referred to as "W promoter (Wp)-restricted" latency because viral gene expression is driven from the Wp, rather than the Q promoter (Kelly *et al.*, 2009). Compared to other BL cells, Wp-restricted BL cells are less sensitive to apoptosis due to the downregulation of Bim (a pro-apoptotic molecule)

by EBNA3A and EBNA3C, and the over-expression of BHRF1 (Anderton *et al.*, 2008; Kelly *et al.*, 2009; Rowe *et al.*, 2009).

2.3.3 EBV infection in asymptomatic hosts

EBV is transmitted orally; infectious virus is shed at low levels in oropharyngeal secretions. Upon initial infection, EBV infects B lymphocytes within the oropharyngeal mucosa and eventually resides mainly in the long-lived memory B cells of asymptomatic carriers (Babcock *et al.*, 1998). However, the mechanism by which EBV becomes resident in the memory B-cell compartment remains controversial (Roughan *et al.*, 2009). One model suggests a direct infection of memory B-cells with EBV (Kurth *et al.*, 2003; Kurth *et al.*, 2000). Another model proposes that EBV infects naïve B cells to become proliferating blasts in which type III latency genes are expressed ("growth programme"). Many of these proliferating cells are eliminated by the primary T-cell response, but some escape and enter the germinal centre where type II latency ("default programme") is established (Babcock *et al.*, 2000b; Roughan *et al.*, 2009). The latently infected cells are subsequently driven into a stable reservoir of resting memory B cells in the peripheral circulation where the expression of all EBV proteins is suppressed ("latency programme" or latency 0) and life-long infection is established (Babcock *et al.*, 2000a). When the latently infected B cells divide to maintain memory B-cell homeostasis, EBNA1 is expressed (type I latency) (Hochberg *et al.*, 2004; Thorley-Lawson *et al.*, 2004). The differentiation of memory B cells into plasma cells triggers the viral replication cycle, possibly at the oropharyngeal epithelium and this releases virions for transmission to new hosts (Thorley-Lawson *et al.*, 2004). A summary of primary EBV infection is illustrated in Figure 2.3.

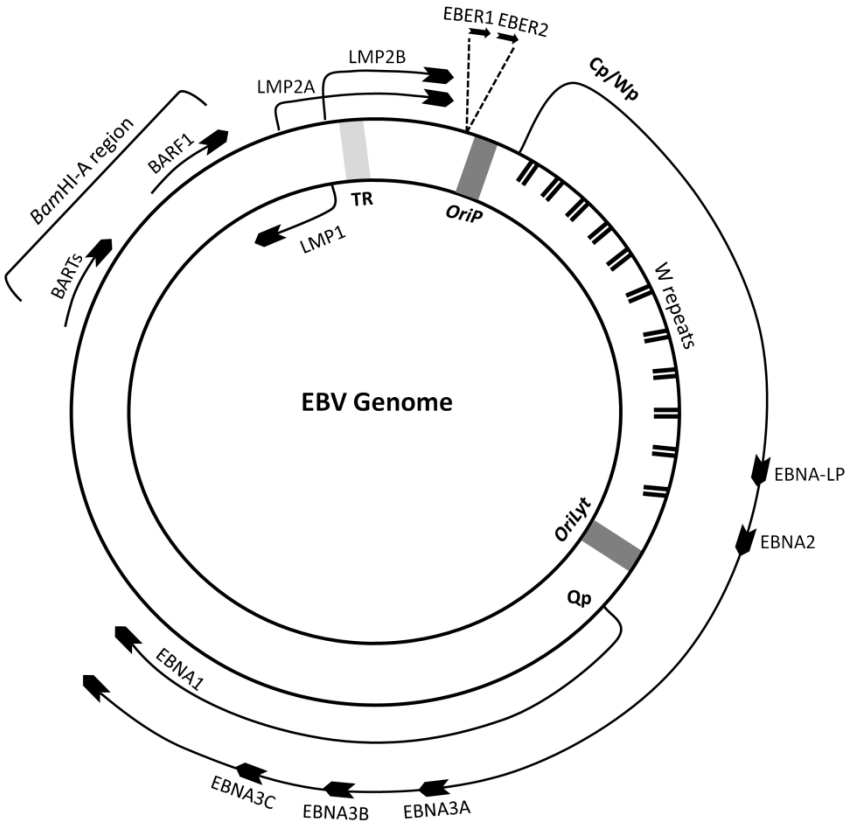


Figure 2.2: The EBV genome

(A) Diagram showing the position and transcription of the EBV latent genes on the double-stranded viral DNA episome with the origins of replication, *oriP* (latent cycle) and *oriLyt* (lytic cycle) indicated. The solid rocket head arrows represent the coding exons for EBV latent proteins and the direction of transcription. The latent proteins include six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and three latent membrane proteins (LMPs 1, 2A and 2B). EBNA-LP is transcribed from variable numbers of repetitive exons in the *Bam*HI W fragment. LMP2A and LMP2B are composed of multiple exons located in the terminal repeat (TR) region, which is generated following circularisation of the linear DNA via fusion of terminal repeats. The long outer line represents the EBV transcripts in latency III where all the EBNAs are transcribed from either C promoter (Cp) or W promoter (Wp) whereas the short inner line shows the EBNA1 transcript originating from Q promoter (Qp) during latency I and latency II. The locations of highly transcribed small non-polyadenylated RNAs, EBER1 and EBER2 are shown here. *Bam*HI-A rightward transcripts (BARTs) and *Bam*HI-A rightward open reading frame 1 (BARF1) are located in the *Bam*HI-A region.

Figure modified from Young & Rickinson, 2004.

Table 2.1: EBV gene expression patterns in different types of latency

EBV latency	EBV gene expression	Examples
Type 0	EBERs	Resting memory B cells
Type I	EBNA1, EBERs, BARTs	Burkitt's lymphoma
Type II	EBNA1, LMP1, LMP2, EBERs, BARTs, BARF1	Nasopharyngeal carcinoma, Hodgkin's lymphoma, gastric carcinoma, extranodal natural killer (ENK)/T-cell lymphoma
Type III	EBNA1, EBNA2, EBNA3, EBNA-LP, LMP1, LMP2, EBERs, BARTs, BHRF1 miRNAs	Lymphoblastoid cell lines, post-transplant lymphoproliferative disorders in immunodeficiency patients

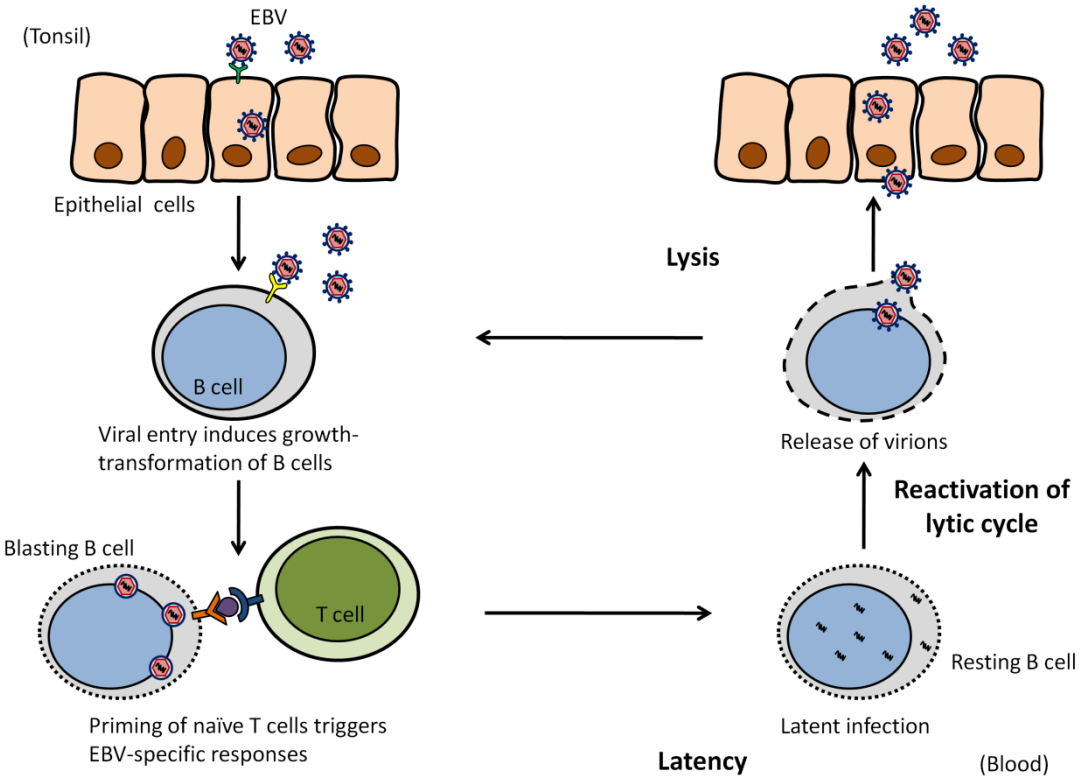


Figure 2.3: EBV infection in healthy virus carriers

Primary EBV infection begins in the tonsil compartment. EBV entry into B cells triggers the B-cell growth programme, leading to the proliferation of blasting B cells. In parallel, priming of naïve T cells by antigen-presenting cells occurs and many of the blasting B cells are destroyed by cytotoxic T lymphocytes. B cells that escape the T-cell response undergo a series of viral latency programme and eventually establish a stable reservoir of resting memory B cells in the blood circulation. Resting memory B cells are activated when differentiating into plasma cells and this induces viral lytic replication and shedding at the oropharyngeal epithelium.

Figure modified from Odumade *et al.*, 2011.

2.3.4 EBV entry mechanisms in B cells and epithelial cells

EBV can infect both B cells and epithelial cells, but through different mechanisms. EBV enters B cells through the attachment of the viral envelope glycoprotein, gp350/220, to the complement receptor 2 (CR2/CD21) or CD35 on the surface of B cells (Nemerow *et al.*, 1985; Ogembo *et al.*, 2013). This brings the virus closer to the cell membrane where another viral glycoprotein, gp42, interacts with the cellular HLA class II molecules, HLA-DR, -DP and -DQ (Haan *et al.*, 2000; Li *et al.*, 1997). The gp42 also bind directly to gH, one of the components of the "core fusion machinery" which consists of a homotrimer gB and a heterodimer gHgL (Hutt-Fletcher, 2007; Li *et al.*, 1995; Ogembo *et al.*, 2013; Wang *et al.*, 1998a). These interactions activate the core fusion machinery leading to the fusion of the virion envelope to the cellular plasma membrane (Chesnokova *et al.*, 2014; Haan *et al.*, 2000; Li *et al.*, 1997).

There is evidence to suggest that EBV can replicate in epithelial cells (Temple *et al.*, 2014). However, EBV infection of human epithelial cells *in vitro* is much less efficient as epithelial cells express neither CR2 nor HLA class II molecules (Hutt-Fletcher, 2007). A number of studies have shown that EBV enters epithelial cells through the interaction of viral gH/gL with the host integrin complexes, αvβ5, αvβ6 and αvβ8, on the cell membrane (Chesnokova *et al.*, 2011; Chesnokova *et al.*, 2009). Additionally, the viral membrane protein BMRF2 has been implicated in EBV attachment to polarised epithelial cells by binding to host β1 integrins (Tugizov *et al.*, 2003; Xiao *et al.*, 2008). Recently, two cellular molecules, neuropilin 1 and nonmuscle myosin heavy chain IIA, have been identified as EBV entry factors into epithelial cells by interacting with gH/gL complex (Wang *et al.*, 2015; Xiong *et al.*, 2015). Furthermore, a novel "in-cell infection" mechanism for EBV infection of nasopharyngeal epithelial cells was described. This process occurs through the invasion of EBV-positive B cells into

epithelial cells by forming cell-in-cell structures that subsequently results in the release of virus into epithelial cells (Ni *et al.*, 2015).

Interestingly, an EBV strain that lacks gp42 cannot infect B cells, but the expression of gp42 impedes EBV infection of epithelial cells (Wang *et al.*, 1998a; Wang *et al.*, 1998b). It has been shown that EBV virions emerged from B cells lack gp42 and that aids EBV entry into epithelial cells while the virions released from epithelial cells are rich in gp42 that facilitate the infection of B cells (Borza *et al.*, 2002). This dual cell tropism plays a critical role for EBV to shuttle between B cells and epithelial cells for the establishment of persistent infection in humans (Chesnokova *et al.*, 2014).

2.3.5 *In vitro* and *in vivo* models of EBV epithelial infection

The ability of EBV to transform B lymphocytes into LCL *in vitro* has greatly facilitated the investigation of the viral transformation mechanisms in B cell tumours. However, one of the hurdles to establish truly representative NPC cell lines is retention of the EBV genome, which is commonly lost in culture (Chang *et al.*, 1989; Glaser *et al.*, 1989; Huang *et al.*, 1980). In addition, epithelial cells are relatively refractory to EBV infection *in vitro* (Imai *et al.*, 1998; Takada, 2000). Early studies attempted to increase EBV infection rates in epithelial cells by stably expressing CR2 but this often resulted in spontaneous lytic reactivation rather than persistent latent infection (Knox *et al.*, 1996; Li *et al.*, 1992). In 1998, successful infection of epithelial cells *in vitro* was achieved by cell-to-cell contact between epithelial cells and recombinant EBV-producing BL-derived Akata cells (Imai *et al.*, 1998). The production of the viruses in Akata cells can be induced by cell surface immunoglobulin G (IgG) cross-linking (Shimizu *et al.*, 1996; Takada, 1984) and the recombinant EBV carries an antibiotic resistance marker that allows the selection of successfully infected-epithelial cells (Chang *et al.*, 1999). Since then, this protocol has been commonly used to establish

EBV-infected NPC cell lines (Tsao *et al.*, 2012). Although the cell-to-cell contact method was successfully used to establish stable EBV infection in NPC cell lines, low EBV infection rates and rapid loss of EBV genome were still observed in non-malignant nasopharyngeal epithelial cells (Tsang *et al.*, 2012; Tsang *et al.*, 2010). It was found that stable EBV infection could only be established *in vitro* in nasopharyngeal epithelial cells harbouring genetic alterations such as overexpression of cyclin D1 and deletion of p16 (Tsang *et al.*, 2012).

Due to the anatomical difference between humans and mice, orthotopic mouse models of NPC are not achievable. As an alternative, a number of EBV-positive patient-derived xenografts (PDXs) have been successfully established as *in vivo* models for NPC. These xenograft models were generated by transplanting the tumours from patients into athymic nude mice or severe combined immunodeficiency (SCID) mice and then re-implanting into other mice to propagate the tumour cells (Morton *et al.*, 2007). Several EBV-positive NPC PDXs (e.g. C15, C17, xeno-2117 and xeno-666) are commonly used by the NPC research community and these PDXs are able to maintain the original tumour histological characteristics, representing useful resources for the study of EBV transformation mechanisms in nasopharyngeal epithelial cells (Bernheim *et al.*, 1993; Busson *et al.*, 1988; Huang *et al.*, 1989; Tentler *et al.*, 2012; Wong *et al.*, 2012).

2.3.6 Functions of EBV latent genes in NPC

2.3.6.1 EBNA1

EBNA1 is consistently found in all types of latency in EBV-associated malignancies (Young *et al.*, 1988). It acts as a sequence-specific DNA binding protein which is responsible for the persistence of EBV genome in latently infected cells by governing the replication and maintenance of the genome (Yates *et al.*, 1985). EBNA1 can also act as a transcriptional transactivator to regulate its own expression and that of other EBV latent genes (LMP1 and C promoter-initiated EBNAs) (Gahn *et al.*, 1995; Schlager *et al.*, 1996; Sugden *et al.*, 1989).

Several studies have reported the ability of EBNA1 to induce oncogenesis in NPC. Expression of EBNA1 was initially found to induce B cell lymphoma in transgenic mice (Wilson *et al.*, 1996) and similarly, expression of EBNA1 in NPC cells significantly promoted tumour formation and metastases following transplantation into nude mice (Sheu *et al.*, 1996). Furthermore, EBNA1 binds to ubiquitin-specific protease 7 and disrupts promyelocytic leukaemia (PML) nuclear bodies that are important for cells to repair DNA damage (Sivachandran *et al.*, 2010; Sivachandran *et al.*, 2008). Consequently, EBNA1 enhances NPC cell survival with DNA damage by impairing p53 activation, apoptosis and DNA repair (Sivachandran *et al.*, 2010; Sivachandran *et al.*, 2008). EBNA1 can also promote genetic instability in NPC cells by inducing DNA damage, reactive oxygen species and upregulating the expression of oxidative stress response proteins SOD1 and Prx1 (Cao *et al.*, 2012). In addition, EBNA1 contributes to angiogenesis by increasing the activity of AP-1 transcription factor, leading to an elevated expression of IL8, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) (O'Neil *et al.*, 2008). Moreover, EBNA1 has been shown to increase the levels of several proteins involved in metastases, including stathmin 1, maspin and Nm23-H1 (Cao *et al.*, 2012). A role for EBNA1 in maintaining EBV

latency was also demonstrated by its ability to induce host let-7 miRNAs which, in turn, reduces the levels of Dicer, a protein that promotes EBV reactivation (Mansouri *et al.*, 2014).

2.3.6.2 LMP1

LMP1 is an integral membrane protein comprising a short cytoplasmic N-terminus, six hydrophobic transmembrane domains and a long cytoplasmic C-terminal tail (Fennewald *et al.*, 1984). LMP1 is a classic oncogene that is able to induce transformation of rodent fibroblasts *in vitro* and tumour formation in nude mice (Wang *et al.*, 1985). LMP1 acts as a constitutively activated member of the tumour necrosis factor receptor (TNFR) superfamily. It activates several signalling pathways including JNK/p-38, PI3K/AKT, MAPK/ERK, NF-κB and JAK/STAT, mainly via the two domains of its cytosolic C-terminus, C-terminal activation region 1 and 2 (CTAR1 and CTAR2), that collectively contribute to a range of tumour-promoting effects (Dawson *et al.*, 2012). For example, LMP1 can promote the growth of NPC cells by upregulating growth factors or the receptors such as insulin-like growth factor 1 (IGF-1), epidermal growth factor receptor (EGFR) and c-Met (Horikawa *et al.*, 2001; Miller *et al.*, 1995; Tworkoski *et al.*, 2015), as well as inhibiting cell cycle negative regulators, such as p16 and p21 (Lo *et al.*, 2004a). A key transforming ability of LMP1 is to modulate cell morphology and promote tumour metastasis. It has been shown that LMP1 can regulate the expression of many molecules that are involved in cell migration and invasion, including matrix metalloproteinase 9 (MMP9), cell division cycle 42 (Cdc42) and tumour necrosis factor alpha-induced protein 2 (TNFAIP2) (Chen *et al.*, 2014; Liu *et al.*, 2012a; Takeshita *et al.*, 1999). Furthermore, LMP1 can induce epithelial-mesenchymal transition (EMT) in NPC (Horikawa *et al.*, 2007; Horikawa *et al.*, 2011) and stimulate cancer stem/progenitor-like phenotype, possibly through the activation of Hedgehog signalling (Kondo *et al.*, 2011; Port *et al.*, 2013). In addition, LMP1 can

stimulate the secretion of pro-inflammatory cytokines in NPC cells, including IL-1α/β and tumour necrosis factor alpha (TNF-α) (Busson *et al.*, 1987; Huang *et al.*, 2010; Morris *et al.*, 2008), suggesting a possible role in modulating the tumour microenvironment. Recent reports also revealed LMP1 is involved in energy metabolism of NPC cells by inhibiting the tumour suppressive liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) pathway or promoting the aerobic glycolysis pathway (Lo *et al.*, 2015; Lo *et al.*, 2013; Xiao *et al.*, 2014). Despite LMP1 exerting an array of effects that favour carcinogenesis, the expression of LMP1 in NPC appears to be variable and heterogeneous, with only approximately 50% of NPC cases staining positive for LMP1 by immunohistochemistry (Chen *et al.*, 2015; Tsao *et al.*, 2002a).

2.3.6.3 LMP2

The LMP2 gene encodes two mRNA products, LMP2A and LMP2B, which are transcribed across the fused terminal repeats of the EBV episome from two different promoters (Sample *et al.*, 1989). LMP2A and LMP2B are highly similar in structure with 12 hydrophobic transmembrane spanning regions and a cytosolic C-terminus, but only LMP2A contains the immunoreceptor tyrosine-based activation motif (ITAM) within its N-terminal cytoplasmic domain that is responsible for many functional effects of LMP2 (Dawson *et al.*, 2012; Raab-Traub, 2002; Sample *et al.*, 1989; Tsao *et al.*, 2015). LMP2A has been reported to stimulate anchorage-independent growth, promote cell migration and invasion, and inhibit cell differentiation of NPC cells. Many of these effects are found to be the consequences of LMP2A-mediated activation of PI3K/AKT and β-catenin signalling (Fotheringham *et al.*, 2012; Fukuda *et al.*, 2007; Lan *et al.*, 2012; Morrison *et al.*, 2004; Morrison *et al.*, 2005; Scholle *et al.*, 2000). The oncogenic effects of LMP2A have also been confirmed *in vivo* where the inoculation of LMP2A-expressing cells in nude mice induced poorly differentiated tumours with high proliferative and metastatic potential (Scholle *et al.*, 2000). Furthermore, LMP2A can

promote EMT, which is related to the acquisition of stem cell-like characteristics in NPC cells, via the induction of metastatic tumour antigen 1 (MTA1) through the mTOR pathway (Kong *et al.*, 2010; Lin *et al.*, 2014c). A novel feature of LMP2A was revealed recently in which LMP2A and LMP1 cooperate to modulate DNA damage signalling to provide a survival advantage for NPC cells (Wasil *et al.*, 2015). Unlike LMP1, the expression of LMP2A is more consistent in NPC (Young *et al.*, 2014). The expression of *LMP2A* mRNA is detected in more than 98% of NPC cases (Brooks *et al.*, 1992; Busson *et al.*, 1992) but LMP2A protein has only been confirmed in approximately 50% of NPC tumour samples (Kong *et al.*, 2010). Low titres of IgG against LMP2A are present in the serum of the majority of the NPC patients, indicating that LMP2A protein is indeed present in NPC (Paramita *et al.*, 2011). Therefore, the low detection rate of LMP2A protein in NPC samples might be an underestimate due to the insensitivity of the anti-LMP2A antibody. While the function of LMP2B remains elusive, it has been shown that both LMP2A and LMP2B can modulate interferon (IFN) signalling by increasing the turnover of IFN receptors and thus limiting the anti-viral response against EBV-infected cells (Shah *et al.*, 2009).

2.3.6.4 EBERs

EBERs are the most abundantly expressed viral transcripts in latently EBV-infected cells. EBERs exist as EBER1 and EBER2 which are non-polyadenylated (non-coding) RNAs that form double-stranded RNA (dsRNA)-like structures (Glickman *et al.*, 1988; Rosa *et al.*, 1981). EBERs are shown to induce the production of type-I IFN through interaction with retinoic acid–inducible gene 1 (RIG-1) (Samanta *et al.*, 2006) or activation of toll-like receptor 3 (TLR3) (Iwakiri *et al.*, 2009), suggesting their involvement in activating the innate immunity. On the other hand, EBERs counteract the effects of IFN by directly binding and inhibiting dsRNA-activated protein kinase R (PKR), protecting EBV-infected cells from IFN-induced Fas-mediated apoptosis

(Nanbo *et al.*, 2002; Nanbo *et al.*, 2005; Yamamoto *et al.*, 2000). EBERs have also been shown to stimulate the secretion of IGF-1 that contributed to the autocrine growth of EBV-infected NPC cells (Iwakiri *et al.*, 2005).

2.3.6.5 Transcripts of *Bam*HI-A region

Two transcripts are encoded from the *Bam*HI A fragment of the EBV genome, namely BARTs and BARF1. BART transcripts are consistently detected in NPC biopsies, suggesting its important role in the development of NPC (Brooks *et al.*, 1993; Tsang *et al.*, 2015). BARTs encode two clusters of miRNAs comprising 44 mature miRNAs that are derived from 22 precursors (Cai *et al.*, 2006; Chen *et al.*, 2010; Grundhoff *et al.*, 2006; Pfeffer *et al.*, 2004). BART miRNAs contribute to the pathogenesis of NPC by regulating a number of viral and cellular genes. BART miRNAs has been shown to negatively regulate LMP1 and LMP2A proteins as well as reduce the sensitivity of LMP1-expressing cells to cisplatin (Lo *et al.*, 2007). Moreover, BART miRNAs maintain viral latency by targeting viral lytic genes (e.g. *BZLF1*, *BRLF1* and *BALF5*) (Barth *et al.*, 2008; Iizasa *et al.*, 2010; Jung *et al.*, 2014; Lung *et al.*, 2009), suggesting that these miRNAs contribute to the immune evasion by NPC cells. Compared to the viral gene targets, more cellular targets of the BART miRNAs have been identified. Importantly, BART miRNAs contribute to cell survival by targeting pro-apoptotic proteins, including p53 up-regulated modulator of apoptosis (PUMA) (Choy *et al.*, 2008), Bim (Marquitz *et al.*, 2011), translocase of outer mitochondrial membrane 22 (TOMM22) (Dolken *et al.*, 2010), caspase 3 (Vereide *et al.*, 2014) and Bid (Shinozaki-Ushiku *et al.*, 2015). Furthermore, BART miRNAs can enhance the growth of NPC cells by suppressing the activity of the tumour suppressor, DICE1, (Lei *et al.*, 2013b) and increase NPC cell migration and invasion by inhibiting the expression of E-cadherin (Hsu *et al.*, 2014). Recent reports have also shown that BART miRNAs can drive tumour growth *in vivo* (Cai *et al.*, 2015; Qiu *et al.*, 2015),

further consolidating the contribution of these miRNAs to EBV oncogenesis. Due to their stability in serum, BART miRNAs have also been recognized as potential diagnostic and prognostic markers for NPC (Chan *et al.*, 2012; Wong *et al.*, 2012).

BARF1 is a homologue of human colony stimulating factor 1 receptor (CSF1R) (Strockbine *et al.*, 1998). *BARF1* was initially thought to be a lytic gene as its expression was induced following the induction of lytic cycle in BL cell lines and the expression of *BARF1* in a high proportion of NPC tissues was suggested to be a consequence of some cells undergoing spontaneous induction of the lytic cycle (Decaussin *et al.*, 2000; Hayes *et al.*, 1999; Zhang *et al.*, 1988). It was later shown in primary NPC tissues that *BARF1* was expressed in the absence of lytic gene expression, pointing to a role for *BARF1* as a latent gene (Seto *et al.*, 2005). Several reports have revealed the oncogenic potential of BARF1 by its ability to induce malignant transformation of rodent fibroblasts and increase NPC cell proliferation and survival *in vitro* as well as enhance tumorigenicity in mouse models (Seto *et al.*, 2008; Sheng *et al.*, 2003; Wei *et al.*, 1994; Wei *et al.*, 1989). The combination of quantitative analysis of EBV DNA and detection of *BARF1* mRNA in nasopharyngeal brushing samples has also been suggested as a useful, non-invasive and sensitive test for the diagnosis of NPC (Stevens *et al.*, 2006).

2.4 Sphingosine-1-phosphate

Sphingolipids are essential components of the lipid bilayer in cellular membranes and provide a protective barrier by giving structural support and mechanical stability (Ogretmen *et al.*, 2004). Many sphingolipids including ceramide, sphingosine, ceramide-1-phosphate, glycosylceramide, lyso-sphingomyelin and S1P are also bioactive lipids that are involved in signal transduction and regulate multiple cellular

processes such as cell growth, survival, senescence, migration, invasion, inflammation, angiogenesis and intracellular trafficking (Hannun *et al.*, 2008; Ogretmen *et al.*, 2004).

S1P ($C_{18}H_{38}NO_5P$) is a zwitterionic lysophospholipid which consists of a serine headgroup with additional dihydrogen phosphate and ammonium radical groups (Rosen *et al.*, 2009). Emerging evidence has revealed the involvement of deregulated S1P signalling in a number of diseases, including cancer, multiple sclerosis, atherosclerosis, diabetes, osteoporosis, sickle cell disease and acute lung injury (Maceyka *et al.*, 2012; Proia *et al.*, 2015).

2.4.1 Metabolism of S1P

Cellular S1P levels are tightly regulated by the balance between its synthesis and degradation (Takabe *et al.*, 2008) (Figure 2.4). The precursor of S1P, ceramide, can be synthesised *de novo* from serine and palmitoyl-CoA or hydrolysed from sphingomyelin. Ceramide is then degraded by ceramidase to produce sphingosine, which is subsequently converted to S1P by sphingosine kinases (SPHKs). S1P can either be reversibly dephosphorylated back to sphingosine by S1P phosphatase or irreversibly cleaved to hexadecenal and ethanolamine phosphate by S1P lyase (Pyne *et al.*, 2000). The balance between ceramide/sphingosine and S1P levels, termed ceramide-sphingosine-S1P rheostat or sphingolipid rheostat, determines cell fate where sphingosine and ceramide are associated with growth arrest and apoptosis, whereas S1P promotes cell proliferation and survival (Milstien *et al.*, 2006).

A wide variety of stimuli, including cytokines (e.g. TNF-α, TGF-β) (Xia *et al.*, 1999; Yamanaka *et al.*, 2004), growth factors (e.g. EGF, IGF, VEGF) (El-Shewy *et al.*, 2006; Hait *et al.*, 2005; Sarkar *et al.*, 2005; Shu *et al.*, 2002), GPCR agonists [e.g. N-formyl-Met-Leu-Phe (FMLP)] (Alemany *et al.*, 1999), hormones (e.g. estradiol) (Sukocheva *et al.*, 2003), phorbol esters [e.g. phorbol 12-myristate 13-acetate (PMA)] (Buehrer *et al.*,

1996) and vitamin D_3 (Kleuser *et al.*, 1998) can activate SPHKs. Two isoforms of SPHKs, SPHK1 and SPHK2, have been identified. The isoforms share approximately 50% identity and 80% similarity and produce S1P, but they are expressed in different cellular compartments and possibly have distinct roles (Liu *et al.*, 2000; Takabe *et al.*, 2008). SPHK1 is predominantly cytosolic; SPHK1 is principally phosphorylated by ERK1/2 (Pitson *et al.*, 2003) and then translocates to the plasma membrane in a calcium and integrin-binding protein 1 (CIB1)-dependent manner to produce S1P (Jarman *et al.*, 2010). In contrast, depending on the cell type, SPHK2 is localised within different intracellular compartments, including endoplasmic reticulum, mitochondria and nuclei (Maceyka *et al.*, 2012). S1P can shuttle between the cytosol and nucleus, an event that appears to be modulated through the phosphorylation by protein kinase D to export nuclear SPHK2 to the cytosol (Ding *et al.*, 2007) and the activation of SPHK2 is also thought to be mediated by ERK-dependent phosphorylation (Hait *et al.*, 2007).

S1P is produced and secreted from various cell types, including epithelial cells (Johnson *et al.*, 2002), cerebellar granule cells, cerebellar astrocytes (Anelli *et al.*, 2005), thrombocytes (Hanel *et al.*, 2007), platelets (English *et al.*, 2000), mast cells, dendritic cells and macrophages (Goetzl *et al.*, 2004). Early studies reported that S1P was abundantly stored in platelets due to their high SPHK activity and absence of S1P lyase (Yatomi *et al.*, 1997a; Yatomi *et al.*, 1997b). It was later found that although erythrocytes have much weaker SPHK activity compared to platelets, they are the major storage sites of S1P, as they are much more abundant in blood and also lack the S1P-degrading enzymes (Hanel *et al.*, 2007; Ito *et al.*, 2007). S1P is regularly released from these storage sites and thereby contribute to a high S1P concentration in the circulation (Hanel *et al.*, 2007; Yatomi *et al.*, 2000). By contrast, S1P levels in most tissues are extremely low because S1P is constantly dephosphorylated by S1P phosphatase or irreversibly degraded by S1P lyase (Rivera *et al.*, 2008). The differences in the levels of

S1P between tissues and blood form a S1P gradient, which is essential for the regulation of various physiological and pathophysiological activities (Hla *et al.*, 2008; Spiegel *et al.*, 2011).

2.4.2 S1P signalling

S1P can act extracellularly through S1P receptors (S1PRs) and/or intracellularly through direct interaction with the intracellular targets (Maceyka *et al.*, 2012).

2.4.2.1 Extracellular action of S1P

S1P is exported out of the cells by transporter proteins (ABCA1, ABCC1, ABCG2 and SPNS2) and acts as a specific ligand for a family of five specific cognate G protein-coupled receptors (GPCR), termed S1PR1 – S1PR5 (Rosen *et al.*, 2005). All five S1PRs consist of an extracellular NH_2 terminus and seven transmembrane domains with different hydrophilic extracellular and intracellular loops according to the receptor subtypes (Sanchez *et al.*, 2004). S1PRs are ubiquitously expressed, albeit at different levels, in many tissues and cell types. S1PR1 (Edg-1), S1PR2 (Edg-5) and S1PR3 (Edg-3) are widely expressed in various tissues, whereas S1PR4 (Edg-6) is confined to lymphoid and hematopoietic tissue and S1PR5 (Edg-8) is found in the central nervous system and NK cells (Sanchez *et al.*, 2004; Takabe *et al.*, 2008).

Extracellular S1P binds S1PRs in a paracrine or autocrine manner to trigger a series of signal transduction pathways that are mediated by different heterotrimeric G proteins, a process termed "inside-out" signalling (Figure 2.5) (Katoh *et al.*, 1998; Takabe *et al.*, 2014). S1PR1 couples only to G_i, S1PR2 and S1PR3 couple to G_i, G_q and $G_{12/13,}$ whereas S1PR4 and S1PR5 couple to G_i and $G_{12/13}$ (Taha *et al.*, 2004). Activation of different G proteins stimulates downstream effectors that may exert entirely different functions in the cells (Wettschureck *et al.*, 2005).

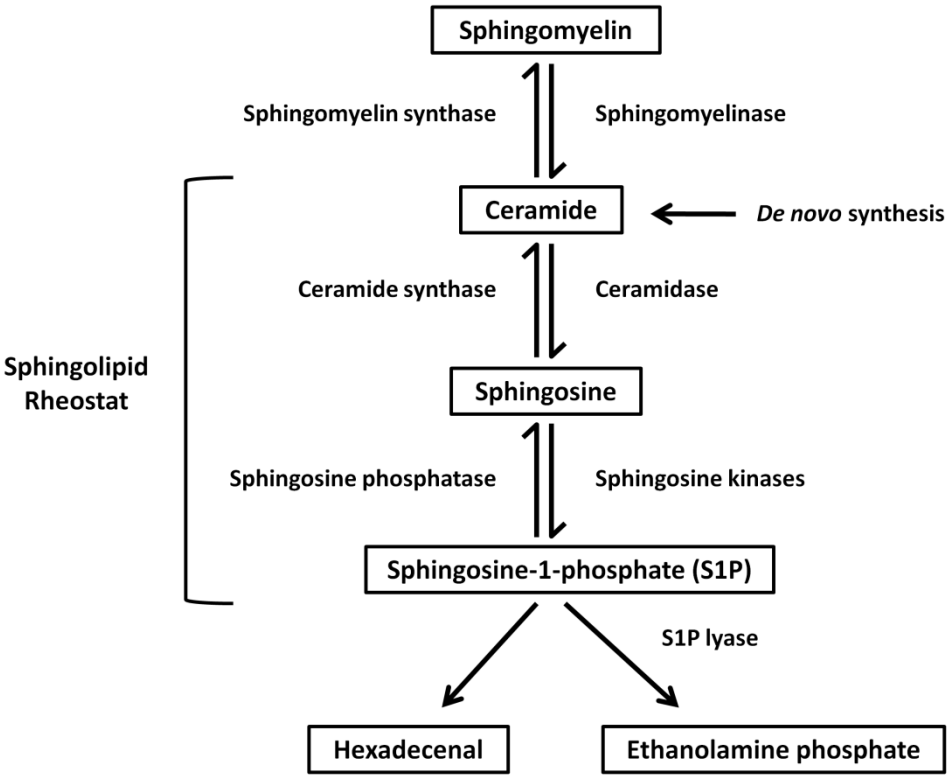


Figure 2.4: Scheme of sphingolipid metabolism

S1P levels are tightly regulated by its synthesis and degradation. Ceramide can be generated through *de novo* synthesis from serine and palmitoyl-CoA or hydrolysis of sphingomyelin. Sphingosine is synthesised from ceramide by ceramidase and subsequently converted to S1P by sphingosine kinases. S1P can be converted back to sphingosine by sphingosine phosphatase or irreversibly cleaved to hexadecenal and ethanolamine phosphate by S1P lyase. The balance between ceramide/sphingosine and S1P levels, termed sphingolipid rheostat, determines cell fate. When the balance shifts towards ceramide and sphingosine, it results in growth arrest and apoptosis. In contrast, when the synthesis of S1P predominates, cell growth and survival are induced.

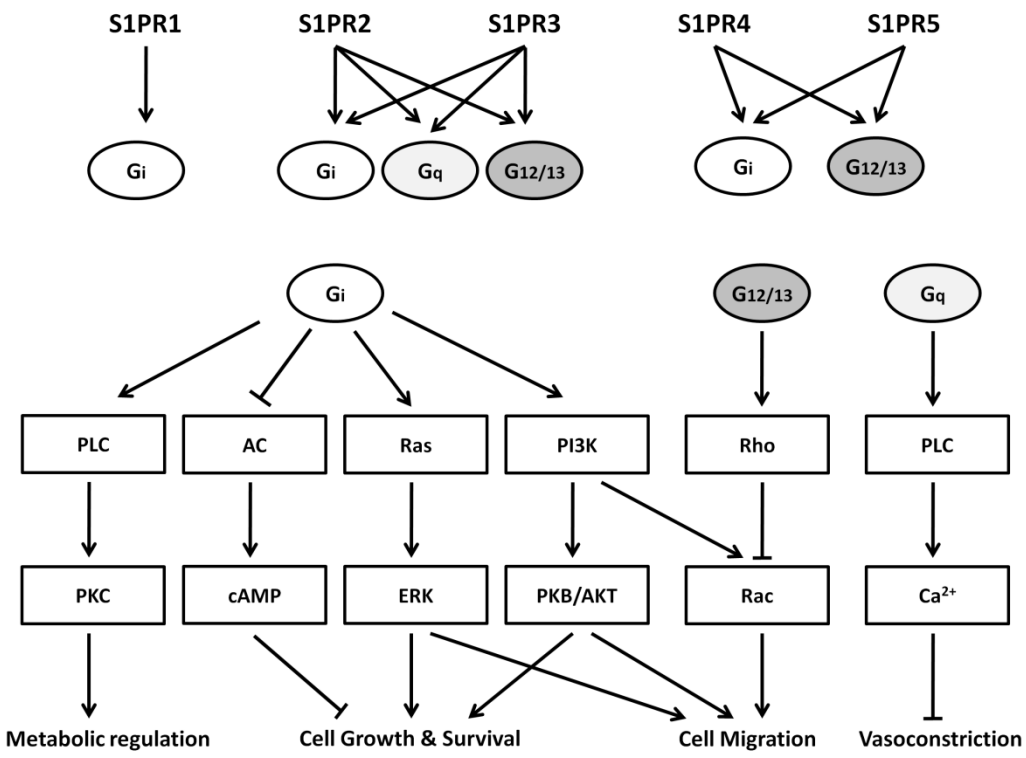


Figure 2.5: S1P receptors, G-protein-coupling and signalling pathways

Binding of S1P to one or more of the five S1PRs activates G proteins (G_i, G_q and $G_{12/13}$). The activated G proteins subsequently trigger downstream signalling pathways and regulate important cellular activities.

PLC, phospholipase C; AC, adenylyl cyclase; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; PKB/AKT, protein kinase B; Ca^{2+}, intracellular free calcium

2.4.2.2 Intracellular action of S1P

Although the majority of the known functions of S1P are attributable to its extracellular action through S1PRs, the lipid can also act as an intracellular second messenger by binding directly to a variety of proteins. These proteins include tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2) (Alvarez *et al.*, 2010), histone deacetylases (HDAC1 and HDAC2) (Hait *et al.*, 2009), β-site amyloid precursor protein cleaving enzyme 1 (BACE1) (Takasugi *et al.*, 2011), prohibitin 2 (PHB2) (Strub *et al.*, 2011). Two additional intracellular targets of S1P were later discovered in 2015, namely peroxisome proliferator-activated receptor γ (PPARγ) (Parham *et al.*, 2015) and human telomerase reverse transcriptase (hTERT) (Panneer Selvam *et al.*, 2015). Although S1P produced by both SPHK1 and SPHK2 can act intracellularly, the intracellular actions of S1P signalling are thought to be mainly attributable to SPHK2-derived S1P, possibly due to its predominant location in intracellular compartments (Spiegel *et al.*, 2011; Zhang *et al.*, 2013).

2.4.3 S1P signalling in cancer

There is a substantial body of evidence to show that aberrant activation of the SPHKs/S1P/S1PRs axis contributes to carcinogenesis. The oncogenic potential of the SPHKs was first revealed in 2000 by the observation that over-expression of SPHK induced the transformation of NIH3T3 fibroblasts and injection of SPHK-overexpressing NIH3T3 cells subcutaneously into NOD/SCID mice resulted in tumour formation (Xia *et al.*, 2000). These studies were performed before the identification of the two SPHK isoforms. Over-expression of SPHK1 was later found in various malignancies and its high expression was correlated to a number of clinicopathological characteristics in various types of cancer, including advanced staging, recurrence and reduced patient survival (Table 2.2) (Pyne *et al.*, 2010). In contrast to SPHK1, the roles of SPHK2 in tumorigenesis are less well-defined.

High expression of SPHKs results in elevated levels of S1P and exogenous addition of S1P promotes the aggressiveness of cancer cells *in vitro*, for example by enhancing cell proliferation, migration and invasion, and by exerting anti-apoptotic effects (Pyne *et al.*, 2010). Furthermore, alterations in the expression or function of one or more of the five S1PRs have been shown to contribute to the oncogenic properties of the S1P signalling pathway (Blaho *et al.*, 2014). Aberrant expression of S1PR1, S1PR2 and S1PR3 has been reported in various types of cancer and several studies have shown an association between the expression of these S1PRs with clinical outcomes, particularly poorer patient survival (Bien-Moller *et al.*, 2016; Go *et al.*, 2015; Lin *et al.*, 2014b; Patmanathan *et al.*, 2016; Watson *et al.*, 2010). The effects of S1P signalling on various cancer cell phenotypes are discussed below.

2.4.3.1 Cell proliferation

The role of S1P in regulating cell proliferation is complex, with evidence for both growth promoting and anti-proliferative effects of the lipid. S1P can promote cancer cell proliferation through the activation of several signalling pathways, including PI3K/AKT and MAPK/ERK (Datta *et al.*, 2014; Goetzl *et al.*, 1999; Nava *et al.*, 2002; Sukocheva *et al.*, 2009; Van Brocklyn *et al.*, 2002; Xia *et al.*, 2012). For example, over-expression of SPHK1 confers a mitogenic advantage to breast cancer cells through an estrogen-dependent mechanism that is associated with the activation of ERK1/2 (Nava *et al.*, 2002). Similarly, silencing of SPHK1 inhibited the proliferation of gastric cancer cells through a mechanism that involves the suppression of AKT activation, as well as stimulation of Forkhead box O1 (FOXO1) activity and the expression of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Xia *et al.*, 2012). On the other hand, several studies have shown that S1P inhibited the growth of cells from a range of tumour types, including gastric, breast, ovarian and thyroid cancers and melanoma (Balthasar *et al.*, 2006; Hong *et al.*, 1999; Ling *et al.*, 2011; Shin *et al.*, 2007; Sultan *et*

al., 2013; Yamashita *et al.*, 2006). However, the precise mechanisms by which S1P inhibits cell proliferation are unclear. The effects of S1P on cancer cell growth are suggested to be influenced by the expression profile of S1PRs (Adada *et al.*, 2013). S1PR1 and S1PR3 are generally associated with increased proliferation of cancer cells (Emery *et al.*, 2014; Hsu *et al.*, 2012; Lee *et al.*, 2010; Liu *et al.*, 2012b), whilst S1PR2 can exhibit either growth promoting (An *et al.*, 2000; Beckham *et al.*, 2013) or suppressive effects (Li *et al.*, 2008b). Interestingly, S1P signalling via S1PR3 has been reported to promote the proliferation of breast cancer stem cells through the activation of Notch signalling (Hirata *et al.*, 2014).

2.4.3.2 Cell migration and invasion

S1P can induce the migration and invasion of cancer cells from various malignancies, including gastric, breast and ovarian cancers and glioblastoma multiforme, via the activation of downstream signalling pathways such as Rac and MAPK/ERK (Arikawa *et al.*, 2003; Bao *et al.*, 2012; Kim *et al.*, 2011; Park *et al.*, 2007; Van Brocklyn *et al.*, 2003; Yamaguchi *et al.*, 2003; Yamashita *et al.*, 2006).

The effects of S1P on the migration and invasion of cancer cells requires the activation of G_i or G_q signalling, suggesting that these effects are also influenced by the expression profile of S1PRs (Van Brocklyn *et al.*, 2003; Wang *et al.*, 1999). Indeed, S1PR1 and S1PR3 have consistently been associated with enhanced migration and invasion of cancer cells (Pyne *et al.*, 2012). For example, coupling with G_i or G_q stimulates the degradation of extracellular matrix by matrix metalloproteinases (e.g. MMP-1, MMP-2, MMP-9) and this occurs mainly via S1PR1 and S1PR3 (Kalhori *et al.*, 2015; Kim *et al.*, 2011; Nyalendo *et al.*, 2007; Van Brocklyn *et al.*, 2003). The migratory and invasive effects of S1PR1 can occur via several downstream mechanisms, for example activation of PI3K and Rac in Wilms tumours (Li *et al.*,

2009a) or interaction with vascular endothelial growth factor receptor 2 (VEGFR-2) that activates ERK1/2 in thyroid cancer cells (Bergelin *et al.*, 2010). In addition, SPHK1/S1P signalling can stimulate the accumulation of ERK1/2 and actin to membrane ruffles/lamellipodia through S1PR3, thereby promoting the migration of breast cancer cells (Long *et al.*, 2010a). The S1P-induced migration of cancer cells can also be mediated via S1PR3 through the activation of HIF-1α, C-reactive protein (CRP) and EGFR (Hsu *et al.*, 2012; Kalhori *et al.*, 2013; Kim *et al.*, 2014). While S1PR2 is generally thought to act as a suppressor of cancer cell migration/invasion via Rho/Rac signalling (Arikawa *et al.*, 2003; Lepley *et al.*, 2005; Malchinkhuu *et al.*, 2008; Yamaguchi *et al.*, 2003), its ability to promote a migratory phenotype has also been reported in various types of cancer *in vitro* and *in vivo* (Li *et al.*, 2015a; Miller *et al.*, 2008; Patmanathan *et al.*, 2016; Ponnusamy *et al.*, 2012; Sekine *et al.*, 2011; Wang *et al.*, 2008).

Table 2.2: Over-expression of SPHK1 in cancer

Cancer type	Clinical parameters associated with high SPHK1 expression	Reference(s)
NPC	Advanced disease stage, locoregional recurrence, distant metastasis and reduced patient survival	(Li *et al.*, 2015b)
HNSCC	Advanced disease stage, nodal involvement, recurrence and poorer patient survival	(Facchinetti *et al.*, 2010; Shirai *et al.*, 2011; Sinha *et al.*, 2011)
Breast cancer	Reduced disease-free survival time and shorter time to recurrence in ER^+ patients	(Long *et al.*, 2010a; Ruckhaberle *et al.*, 2008; Watson *et al.*, 2010)
Astrocytoma	Higher histological tumour grade and shorter patient survival	(Li *et al.*, 2008a; Van Brocklyn *et al.*, 2005)
Esophageal cancer	Increased tumour invasion depth, lymph node metastasis and poorer patient survival	(Pan *et al.*, 2011)
Gastric cancer	Advanced disease stage, increased tumour size, distant metastasis and reduced patient survival	(Li *et al.*, 2009b)
Non-Hodgkin's lymphoma	Increased tumour clinical grade	(Bayerl *et al.*, 2008)
Bladder cancer	Higher histological tumour grade, advanced disease stage and reduced overall 5-year survival rates	(Meng *et al.*, 2014)
Colon cancer	Enhanced tumour metastasis	(Kawamori *et al.*, 2009)
Cervical cancer	Increased tumour size, invasion depth, disease stage, lymph node metastasis and lymphovascular invasion as well as reduced overall survival and recurrence-free survival	(Kim *et al.*, 2015)
Thyroid cancer	Advanced disease stage	(Guan *et al.*, 2011a)

NPC, nasopharyngeal carcinoma; HNSCC, head and neck squamous cell carcinoma; SPHK1, sphingosine kinase; ER, estrogen receptor.

2.4.3.3 Other phenotypes

In addition to cell proliferation and migration/invasion, aberrant S1P signalling has been implicated in regulating other malignant phenotypes, such as enhanced cell survival, angiogenesis and resistance to cancer therapy.

The balance between the levels of ceramide and S1P is critical in regulating cell survival. High expression of SPHK1 leads to elevated levels of S1P with a reduction in ceramide levels and this results in the inhibition of ceramide-mediated apoptosis (Bektas *et al.*, 2005; Cuvillier *et al.*, 2001; Pchejetski *et al.*, 2005). S1P can suppress cancer cell apoptosis mainly via S1PR1 through several mechanisms, including activation of PI3K/AKT, MAPK/ERK, NF-κB pathways and inducing the expression of anti-apoptotic proteins of the BCL-2 family (e.g. Bcl-2, Bcl-xL, Mcl-1) (Bektas *et al.*, 2005; Kapitonov *et al.*, 2009; Li *et al.*, 2007; Li *et al.*, 2008c; Song *et al.*, 2011; Xu *et al.*, 2016), as well as inhibition of pro-apoptotic proteins (e.g. Bim, Bax) (Guan *et al.*, 2011b; Taha *et al.*, 2006a).

SPHK1/S1P signalling has been shown to induce tumour angiogenesis and lymphangiogenesis *in vitro* and/or *in vivo* in models of glioblastoma multiforme, as well as lung and breast cancers (Anelli *et al.*, 2010; Chae *et al.*, 2004; Kapitonov *et al.*, 2009; Nagahashi *et al.*, 2012). Increased expression of S1PR1 and S1PR3, and decreased expression of S1PR2 have been reported to be critical in mediating these effects (Chae *et al.*, 2004; Du *et al.*, 2010; Yoon *et al.*, 2008). The S1P-induced angiogenesis and lymphangiogenesis in cancer cells have also been correlated to elevated expression of *VEGF* and HIF-1α (Ader *et al.*, 2015; Anelli *et al.*, 2010; Chae *et al.*, 2004; Kalhori *et al.*, 2013; Li *et al.*, 2011).

Another oncogenic function of S1P is increased resistance of cancer cells to chemotherapeutic drugs and γ-irradiation and these effects appear to be mediated

through S1PR1, S1PR2 and S1PR3 (Akao *et al.*, 2006; Pyne *et al.*, 2010; Salas *et al.*, 2011; Watson *et al.*, 2010). High expression of SPHK1 correlated with a shorter time to recurrence in ER^+ breast cancer patients receiving tamoxifen, indicating the induction of tamoxifen resistance (Long *et al.*, 2010a). Furthermore, cancer cell lines that express high levels of SPHK1 are resistant to gemcitabine, camptothecin, tamoxifen and cetuximab (Akao *et al.*, 2006; Guillermet-Guibert *et al.*, 2009; Pchejetski *et al.*, 2005; Rosa *et al.*, 2012; Sukocheva *et al.*, 2009). Over-expression of SPHK1 has also been shown to reduce sensitivity of tumour cells to chemotherapeutic drugs and radiation therapy *in vivo* (Pchejetski *et al.*, 2005) (Sinha *et al.*, 2011).

2.4.3.4 *In vivo* studies

Several key *in vivo* studies have confirmed the importance of aberrant S1P signalling in promoting tumorigenesis For example, knockout of SPHK1 in a mouse model of head and neck cancer decreased S1P formation and suppressed 4-nitroquinoline 1 oxide (4-NQO)-induced carcinogenesis through reduced tumour incidence, multiplicity and volume that was associated with decreased growth, survival and phosphorylation of AKT (Shirai *et al.*, 2011). In addition, S1P enhances tumour growth and metastasis *in vivo* in the models of bladder cancer and colon cancer through persistent activation of STAT3 via S1PR1 (Lee *et al.*, 2010; Liang *et al.*, 2013). A possible feedback loop was induced in which binding of SPHK1-derived S1P to S1PR1 stimulated secretion of NF-κB-regulated pro-inflammatory cytokine IL-6, leading to the activation of STAT3 that in turn upregulates the expression of S1PR1; decreased expression of SPHK1 and S1PR1 suppressed the NF-κB-IL-6-STAT3 amplification cascade and the development of colitis-associated cancer (Liang *et al.*, 2013). Studies using melanoma mouse models have suggested that S1PR1/STAT3 signalling is also crucial in facilitating the formation of pre-metastatic niches and inducing myeloid cell colonisation at future metastatic sites (Deng *et al.*, 2012).

Collectively, these studies highlight the potential of targeting the SPHK-S1P-S1PR axis for cancer therapeutics. The case for targeting this pathway is even more compelling following the report that SPHK1 expression in cancer-associated fibroblasts (CAFs) promotes the migration and metastatic dissemination of melanoma cells, via the upregulation of S1PR3 (Albinet *et al.*, 2014). Furthermore, these authors show that conditioned media from SPHK1-expressing melanoma cells induces fibroblasts to differentiate into myofibroblasts and recently a critical role was reported for SPHK1 in mediating TGF-β-induced myofibroblast differentiation in ovarian cancer (Beach *et al.*, 2016). Therefore, SPHK1 appears to play a dual role by enhancing tumour cell migration and metastasis, whilst also stimulating the development of tumour promoting CAFs. This raises the exciting possibility that inhibitors of S1P signalling would simultaneously target both malignant cells and the tumour permissive microenvironment.

2.4.4 Therapeutic agents targeting S1P signalling

Targeting the S1P signalling pathway is now considered a promising therapeutic approach for the treatment of cancer. A number of strategies to target different molecules within the pathway have been established, including inhibition of SPHKs, neutralisation of S1P itself and targeting specific S1PRs (Kunkel *et al.*, 2013).

2.4.4.1 Inhibition of SPHKs

Inhibition of SPHKs is a promising approach to reduce the levels of S1P and increase the levels of ceramide and sphingosine. The activity of SPHKs can be modulated using inhibitors, such as SKI-I (specific for SPHK1), PF-543 (selective for SPHK1), Safingol (putative SPHK1 inhibitor), ABC294640 (selective for SPHK2), SKI-II (ski; inhibits SPHK1 and SPHK2) and DMS (inhibits SPHK1 and SPHK2) (Kunkel *et al.*, 2013; Pyne *et al.*, 2010). SPHK inhibitors have been found to be effective in reducing S1P

levels and to inhibit the tumour-promoting effects of S1P both *in vitro* and *in vivo* (Beljanski *et al.*, 2010; French *et al.*, 2006; French *et al.*, 2010; Gao *et al.*, 2011; Kapitonov *et al.*, 2009; Nagahashi *et al.*, 2012; Paugh *et al.*, 2008; Schnute *et al.*, 2012; Sukocheva *et al.*, 2009). Some of these inhibitors are now entering early stage of clinical trials. For example, a phase I clinical trial using Safingol for solid tumours has been completed and the results demonstrated that it can be administered to patients safely in conjunction with cisplatin, although reversible dose-dependent hepatic toxicity was observed (Dickson *et al.*, 2011). Similarly, a phase I clinical trial using ABC294640 (formulated as YELIVA™) for pancreatic cancer and advanced solid tumours has also just been completed and the results showed that the drug is well-tolerated (www.redhillbio.com).

2.4.4.2 Sequestration of S1P

A number of anti-S1P monoclonal antibodies have been developed to neutralise systemic S1P. An early *in vitro* study using an anti-S1P monoclonal antibody has demonstrated its efficacy in inhibiting S1P-mediated cancer progression and angiogenesis in lung, breast and colorectal cancer cell lines (Visentin *et al.*, 2006). The anti-tumorigenic and anti-angiogenic effects of the antibody were also observed in mouse xenograft and allograft models (O'Brien *et al.*, 2009; Ponnusamy *et al.*, 2012; Visentin *et al.*, 2006; Zhang *et al.*, 2015). Two anti-S1P monoclonal antibodies, LT1002 and LT1009 (the humanised form of LT1002), have been reported to have high affinity and specificity for S1P and do not cross-react with other structurally related lipids (O'Brien *et al.*, 2009). LT1009 has been formulated as ASONEP™ by Lpath, Inc. and the Phase I clinical trials using this drug for advanced solid tumours was completed in 2011 (Sabbadini, 2011). However, its Phase II clinical trial for unresectable and refractory renal cell carcinoma was terminated prematurely due to frequent occurrence of serious adverse events (clinicaltrials.gov).

2.4.4.3 Targeting of S1P receptors

Several pharmacological drugs, including FTY720, JTE-013 and VPC23019, have been developed to target the S1PRs, although the majority of studies with these molecules are restricted to preclinical disease models. FTY720 (fingolimod) is a sphingosine analog that can bind and induce endocytosis of four S1PRs (S1PR1, S1PR3, S1PR4 and S1PR5) at concentrations lower than 0.1μM (Brinkmann *et al.*, 2002). This compound was initially found to have immunosuppressive effects by inhibiting the receptor signalling involved in lymphocyte egress (Mandala *et al.*, 2002; Matloubian *et al.*, 2004). These immunosuppressive effects were particularly demonstrated to be mediated by S1PR1 because FTY720 downregulated S1PR1 in lymphocytes resulting in lymphocyte sequestration (Matloubian *et al.*, 2004). FTY720 was later shown to inhibit cell growth, survival, migration and increase chemosensitivity in various types of cancer *in vitro* and *in vivo* (Azuma *et al.*, 2002; Ho *et al.*, 2005; LaMontagne *et al.*, 2006; Matsuoka *et al.*, 2003; Ubai *et al.*, 2007). Formulated as Gilenya™, FTY720 was approved in September 2010 as the first oral treatment for relapsing and remitting multiple sclerosis (Brinkmann *et al.*, 2010), and the re-purposing of this drug for cancer treatment is potentially a promising strategy (Patmanathan *et al.*, 2015).

JTE-013 is a S1PR2/S1PR4 antagonist. It has been shown to be effective in inhibiting S1PR2 at sub-micromolar concentrations (Ohmori *et al.*, 2003; Osada *et al.*, 2002). Blockade of S1PR2 by JTE-013 enhanced the motility of melanoma and glioma cells (Arikawa *et al.*, 2003; Li *et al.*, 2015a) as well as promoted the growth of Wilm's tumour cells (Li *et al.*, 2008b). In contrast, several studies have shown that the migration of cancer cells, including ovarian cancer, prostate cancer and neuroblastoma, was inhibited following JTE-013 treatment (Li *et al.*, 2015a; Miller *et al.*, 2008; Sekine *et al.*, 2011). JTE-013 has also been found to suppress the S1P-induced activation of

ERK1/2 via S1PR4 in breast cancer cells at a concentration of 10μM (Long *et al.*, 2010b).

VPC23019 acts as a competitive inhibitor of S1P binding to S1PR1 and S1PR3 (Davis *et al.*, 2005). The actions of VPC23019 were demonstrated through competitive suppression of S1P-mediated calcium mobilisation in bladder carcinoma cells stably over-expressing S1PR3 and displacement of radiolabeled S1P binding at S1PR1 and S1PR3 in HEK293T cells (Davis *et al.*, 2005). In the context of cancer research, VPC23019 significantly inhibited S1P-induced cell migration of ovarian cancer, prostate cancer, thyroid cancer and liver cancer (Bao *et al.*, 2012; Bergelin *et al.*, 2009; Park *et al.*, 2007; Sekine *et al.*, 2011; Wang *et al.*, 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell lines

Eight NPC cell lines, two immortalised nasopharyngeal epithelial cell lines, five EBV-infected NPC cell lines (CNE2/EBV, HK1/EBV, HONE1/EBV, SUNE1/EBV, TW01/EBV), an EBV-infected immortalised nasopharyngeal epithelial cell line (NP460hTert/EBV), an EBV-positive BL cell line (Akata), an LCL (X50-7) and two human embryonic kidney cell lines (HEK293 and HEK293T) were used in this study. The cell lines were kind gifts from Professor George Tsao, University of Hong Kong; Dr Christopher Dawson, University of Birmingham and Professor Chee Onn Leong, International Medical University. The characteristics and EBV status of NPC cell lines and immortalised nasopharyngeal epithelial cell lines are shown in Table 3.1.

3.2 Materials

S1P (D-erythro S1P, d18:1; Avanti Polar Lipids, USA) was dissolved in 95% methanol (Merck, Germany), dried under a stream of nitrogen gas and stored at -20 ℃ in aliquots of 100nmol. S1P was re-constituted in RPMI medium containing 4mg/ml fatty acid-free albumin (FAFA; Sigma-Aldrich, USA) at 37^{o}C overnight prior to experiments. JTE-013 [1-[1,3-Dimethyl-4-(2-methylethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicarbazide] and VPC23019 (2-Amino-N-(3-octylphenyl)-3-(phosphonooxy)-propanamaide) were obtained from Tocris Biosciences, UK. LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was obtained from Merck, Germany. CYM-5478 was a kind gift from Dr Deron Herr, National University of Singapore. JTE-013, LY294002 and CYM-5478 were dissolved in DMSO and VPC23019 was dissolved in acidified DMSO [5% 1N hydrochloric acid (HCl; Merck, Germany) in DMSO]. All compounds were stored at -20 ℃ in small aliquots.

Table 3.1: Characteristics of the NPC cell lines and immortalised nasopharyngeal epithelial cell lines

Cell Lines	Differentiation	EBV Status	References
C666-1	Undifferentiated	Positive	(Cheung *et al.*, 1999)
CNE1	Well-differentiated	Negative	("Establishment of an epitheloid cell line and a fusiform cell line from a patient with nasopharyngeal carcinoma," 1978)
CNE2	Poorly differentiated	Negative	(Sizhong *et al.*, 1983)
HK1	Well-differentiated	Negative	(Huang *et al.*, 1980)
HONE1	Poorly differentiated	Negative	(Glaser *et al.*, 1989)
SUNE1	Poorly differentiated	Negative	(Chen *et al.*, 1998)
TW01	Well-differentiated	Negative	(Lin *et al.*, 1993)
TW04	Poorly differentiated	Negative	(Lin *et al.*, 1993)
NP460hTert	Non-malignant nasopharyngeal epithelium, immortalized using hTERT	Negative	(Li *et al.*, 2006a)
NP69	Non-malignant nasopharyngeal epithelium, immortalised using SV40T	Negative	(Tsao *et al.*, 2002b)

3.3 Cell culture

3.3.1 Maintenance of cell lines

NPC cell lines, Akata and X50-7 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, USA). NP69 was cultured in keratinocyte serum-free medium (KSFM; Gibco Life Technologies, USA) containing 25μg/ml of bovine pituitary extract (Gibco Life Technologies, USA), 0.2ng/ml of epidermal growth factor (Gibco Life Technologies, USA) and 0.3mM of calcium chloride and NP460hTert was grown in a 1:1 mixture of defined KFSM (Gibco Life Technologies, USA) and Epilife® medium with growth supplements (Cascade Biologics, USA). HEK293 and HEK293T were cultured in *Dulbecco's Modified Eagle's medium* (DMEM) medium (Gibco Life Technologies, USA) supplemented with 10% FBS (Gibco Life Technologies, USA). All cultures were maintained in a 37℃ CO_2 incubator (Binder, Germany) supplied with 5% CO_2.

3.3.2 Sub-culturing and Cell Number Determination

Cells were sub-cultured when they reached 80-90% confluency. To sub-culture adherent cells, cells were washed with PBS (Gibco Life Technologies, USA) and trypsinised with 0.25% trypsin-EDTA (Gibco Life Technologies, USA). An equal volume of complete growth medium was added to neutralize the enzymatic action and cells were pelleted by centrifugation at 1,000rpm for 8 minutes. For suspension cells, cells were directly pelleted by centrifugation at 300rpm for 5 minutes. Cell pellets were re-suspended in fresh medium and the cell number was determined using a Luna automated cell counter (Logos Biosystems, Korea). Cells were mixed 1:1 with Trypan Blue (Gibco Life Technologies, USA) and 10μl of the mixture was pipetted into Luna cell counting chamber (Logos Biosystems, Korea). The number of viable cells was determined and cells were seeded at required densities according to the experimental design.

3.3.3 Cryopreservation and recovery of cells

$1x10^6$ cells were re-suspended in FBS containing 10% DMSO (Sigma-Aldrich, USA) and transferred into cryovials (Nunc, USA). The cryovials were stored in MrFrosty™ Cryo 1℃ Freezing Container (Nalgene, USA) at -80℃ freezer overnight before transferring to liquid nitrogen for long term storage.

Cryopreserved cells were recovered by rapid thawing at 37^oC. Cells were transferred into 9ml complete growth medium and centrifuged at 1,000rpm for 8 minutes. The supernatant was discarded and cell pellet was re-suspended with fresh medium. Cells were grown in a $25cm^2$ flask in 5ml of medium.

3.3.4 Transient transfection of cell lines

Four genes (SPHK1, constitutively active AKT, LMP1 and LMP2A) were transiently expressed in cultured cells using a FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacturer's protocol. Briefly, cells were seeded at appropriate densities to reach 60% confluence prior to the experiments. Cells were washed with PBS and replenished with fresh growth medium. A 3:1 ratio of FuGENE® HD Transfection Reagent and plasmid DNA was added to complete growth medium and incubated at room temperature for 10 minutes. The mixture was added drop-wise to the cells and the cells were incubated for 24-72 hours according to the experimental design. Plasmid DNAs used were pCMV6_XL4/SPHK1, pCDNA3.1/myr-AKT, pCDNA3.1/LMP1 and pSG5/LMP2A.

3.3.5 Knockdown of SPHK1 in NPC cell lines

3.3.5.1 Generation of puromycin kill curves

Cells were seeded in 6-well plates at appropriate densities one day prior to the experiments to obtain a confluency of 60%. Various concentrations of puromycin (0, 0.25, 0.5, 1, 2.5, 5μg/ml) (Fisher Scientific, USA) were added to the cells and incubated

for 4 days. The minimum concentration of puromycin that killed 90% of the cells was used for subsequent experiments.

3.3.5.2 Collection of lentiviral supernatants

Lentiviral vector system was used to knock down SPHK1 in HONE1 and C666-1 cells. 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). $1x10^7$ HEK293T cells were seeded in a $150cm^2$ flask one day before the experiments and cultured until 80-90% confluence. 40μg lentiviral construct, 10μg envelope plasmid (pMD2.G) and 30μg packaging plasmid (psPAX2) were added to 5ml of Opti-MEM® (Gibco Life Technologies, USA) and filtered through a 0.2μm filter (Sartorius, Germany). 1μl of 10mM stock of polyethylenimine (PEI) (Sigma-Aldrich, USA) was added to another 5ml of Opti-MEM® and filtered through a 0.2μm filter. Plasmid DNA and PEI solutions were then mixed at 1:1 ratio and incubated at room temperature for 20 minutes. HEK293T cells were washed with Opti-MEM® and 10ml of the plasmid DNA and PEI complexes were added to the cells. Cells were incubated at 37^oC, 5% CO_2 for 4 hours and the medium was replaced with complete DMEM. The lentiviral supernatant was collected after 48 hours and filtered through a 0.45μm filter. The lentiviral supernatant were stored at -80^oC in aliquots until use.

3.3.5.3 Lentiviral transduction of NPC cells

$3x10^5$ HONE1 cells or $8x10^5$ C666-1 cells were seeded in $25cm^2$ flasks one day before the experiments and cultured until 60% confluence. Lentiviral supernatant was reconstituted with fresh RPMI medium at 1:1 ratio. Polybrene was added to the lentiviral supernatant at a final concentration of 7.5μg/ml. The lentivirus/polybrene mixture was added to cells and incubated at 37^oC, 5% CO_2 for 18 hours. Cells were then

cultured in fresh complete growth medium containing puromycin for a week to select stable transfectants.

3.3.6 Knockdown of S1PR3 in SUNE1 cells

SMARTpool ON-TARGETplus S1PR3 siRNA (LU-005208-00; Dharmacon, USA) and ON-TARGETplus non-targeting siRNA pool (Dharmacon, USA) were re-suspended according to the manufacturer's protocol. Briefly, 1x siRNA buffer (Dharmacon, USA) was added to the siRNAs to acquire a stock concentration of 20μM. The solution was mixed well and placed on an orbital shaker at room temperature for 30 minutes. The tubes were briefly centrifuged and the siRNAs were aliquoted and stored at -80^{o}C until use.

3x10^{5} SUNE1 cells were seeded into 6-well plates one day before the experiments to reach a confluency of 60%. Transfection of cells was performed according to the manufacturer's protocol. Briefly, siRNAs at desired concentrations and DharmaFECT 1 transfection reagent (Dharmacon, USA) were diluted with serum-free medium separately and incubated at room temperature for 5 minutes. The diluted siRNAs were then mixed with the diluted DharmaFECT 1 transfection reagent and incubated at room temperature for 20 minutes. The mixture was added to cells and the cells were incubated at 37^{o}C, 5% CO_2 for 48-72 hours according to the experimental design.

3.4 EBV infection

Successful EBV infection of epithelial cells *in vitro* can be achieved through cell-to-cell contact method by co-culturing epithelial cells with recombinant EBV-producing Akata cells (Chang *et al.*, 1999; Imai *et al.*, 1998). The recombinant EBV carries a neomycin resistance marker, allowing the selection of successfully infected cells (Imai *et al.*, 1998). Two NPC cell lines (CNE1 and TW04) were selected to be stably infected with EBV based on their low levels of SPHK1 and S1PR3. Akata cells were cultured at

a density of $2x10^6$ cells/ml and treated with an anti-human IgG antibody (1:1000 dilution; MP Biomedicals, USA) for 72 hours prior to co-culturing with the NPC cells. $3x10^5$ NPC cells were seeded in 6-well plates one day before the experiments. $1.5x10^6$ anti-IgG-treated Akata cells were added to NPC cells and co-cultivated for 3 days. The Akata cells were then removed and the NPC cells were washed with PBS. The NPC cells were grown in complete RPMI medium containing G418 (Biowest, France) for a week to select stable transfectants.

3.5 *In vitro* assays

3.5.1 Cell proliferation assays

1 – $3x10^3$ cells were seeded in 96-well plates and allowed to adhere overnight. For experiments investigating the effects of exogenous S1P, cells were serum-starved overnight and treated with a range of S1P concentrations (0 – 10μM) for a period of 2 days. At the desired time point, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; 5mg/ml; Merck, Germany) was added to the cells and incubated for 4 hours, followed by the addition of 10% sodium dodecyl sulphate (SDS; Thermo Scientific, USA)/0.01M HCl. After overnight solubilisation of the formazan, cell viability was assessed by measuring the absorbance of the dissolved formazan solution at a wavelength of 575nm with a reference wavelength of 650nm using an Infinite 200 Pro NanoQuant microplate reader (Tecan, Switzerland). To generate kill curves of the pharmacological drugs, cells were treated with a range of drug concentrations (0 – 10μM) for 24 hours.

To determine the proliferation of EBV-infected cells, 2 – $3x10^4$ cells were seeded in 6-well plates and allowed to adhere overnight. Cells in triplicate wells were trypsinized and counted using a Luna automated cell counter as previously described (Section 3.3.2) every day for six consecutive days. The medium was changed every two days.

3.5.2 Transwell migration assays

Transwell migration assays were performed using polycarbonate inserts (8μm pore size, Transwell, Corning, USA) coated with 10μg/ml fibronectin (Gibco Life Technologies, USA). $3x10^5$ cells were grown in a $75cm^2$ flask for two days until 80% confluence. Cells were serum-starved overnight, followed by treatment with 10μg/ml mitomycin C (Merck, Germany) for 2 hours to limit cell proliferation. Transwell inserts were placed in 24-well plates to create upper and lower chambers. For experiments using S1P, $5x10^4$ cells were re-suspended in 200μl migration buffer (RPMI medium containing 0.1% FBS and 0.25mg/ml FAFA) and seeded into the upper chambers; 500μl migration buffer containing S1P (0, 1 or 5μM) was added to the lower chambers. For experiments using pharmacological drugs, migration buffer containing the desired concentrations of the pharmacological drugs was added to both the upper and lower chambers of the Transwell inserts. For experiments using the EBV-infected cells, $5x10^4$ – $1x10^5$ cells in 200μl RPMI medium containing 0.1% FBS were seeded into upper chambers and 500μl RPMI medium containing 10% FBS was added to the lower chambers. The cells were allowed to migrate for 19 hours and cells remained in the upper chambers were removed with cotton buds. Cells migrated through the membrane were stained with 0.1% crystal violet (Merck, Germany) in 20% methanol for 2 hours and counted in five random fields at 20X magnification.

3.5.3 Transwell invasion assays

Cell invasion assays were performed using polycarbonate inserts (8μm pore size, Transwell, Corning, USA) coated with 250μg/ml BD matrigel basement membrane matrix (BD Biosciences, USA). $3x10^5$ cells were grown in a $75cm^2$ flask for two days until 80% confluence. Cells were serum starved overnight and treated with 10μg/ml mitomycin C two hours prior to the experiments. For experiments using S1P, $8x10^4$ cells were re-suspended in 500μl migration buffer and seeded into the upper chambers;

750µl migration buffer containing S1P (0 or 5µM) was added to the lower chambers. For experiments using the EBV-infected cells, 2 – 4x10^5 cells in 500µl RPMI medium containing 0.1% FBS were seeded into upper chambers and 750µl RPMI medium containing 10% FBS was added to the lower chambers. After 48 hours of incubation, the non-invaded cells in the upper chambers were removed with a cotton swab and the invaded cells in the lower chambers were stained and counted as described above (Section 3.5.2).

3.6 Molecular biology

3.6.1 Total RNA isolation

Cells were cultured until 60% confluence and the total RNA was extracted using an RNeasy® mini kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, cells were lysed and the lysate was homogenised using a QIAshredder spin column (Qiagen, Germany). 70% ethanol (Merck, Germany) was added to the flow-through and the mixture was loaded to an RNeasy® spin column. After centrifugation, the spin column membrane was then washed with wash buffer and DNase I incubation mix (RNase-Free DNase Set; Qiagen, Germany) was applied directly to the spin column membrane. Following DNA digestion, wash buffer was added to the column and RNA was eluted in 50µl RNase-free water. The concentration of RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

3.6.2 cDNA synthesis

Single-stranded cDNA was synthesised using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's protocol. RNase-free water was added to 1µg of RNA to a final volume of 10µl. The reverse transcription master mix containing 2µl of 10X RT Random Primers, 2µl of 10X RT Buffer, 0.8µl of 100mM deoxyribonucleotide triphosphates (dNTP) mix, 1µl of

MultiScribe™ Reverse Transcriptase and 4.2µl RNase-free water was added to the RNA sample and mixed well. The tubes were briefly centrifuged and cDNA synthesis was carried out in a thermal cycler (Applied Biosystems, USA) using the following conditions: 25^{o}C for 10 minutes, 37^{o}C for 2 hours, 85^{o}C for 5 minutes and hold at 4^{o}C.

3.6.3 Real time quantitative polymerase chain reaction (Q-PCR)

FastStart Universal Probe *Master* (Rox) was purchased from Roche, Switzerland and the commercially available TaqMan® Gene Expression Assays for SPHK1 (Hs00184211_m1), S1PR1 (Hs01922614_s1), S1PR2 (A139R4J), S1PR3 (Hs00245464_s1), S1PR4 (Hs02330084_s1), S1PR5 (Hs00928195_s1) and GAPDH (4326317E) were purchased from Applied Biosystems, USA. The primer and probe sequences of EBNA1, LMP1 and LMP2A (Table 3.2) were synthesised as previously described (Bell *et al.*, 2006) and all probes were labelled with a FAM reporter dye at 5' end and a TAMRA quencher dye at 3' end. The synthesised cDNA was diluted using nuclease-free water (Gibco Life Technologies, USA) at a 1:20 ratio prior to the experiments. The reaction mixture for Q-PCR using commercially available TaqMan® Gene Expression Assays consisted of 5µl diluted cDNA, 10µl FastStart Universal Probe Master (Rox), 1µl TaqMan® Gene Expression Assay, 1µl human GAPDH primer/probe and 3µl nuclease-free water. The master mix for the detection of EBNA1, LMP1 and LMP2A gene expression consisted of 5µl diluted cDNA, 12.5µl FastStart Universal Probe Master (Rox), 2.5µl 5' primer (3µM), 2.5µl 3' primer (3µM), 1µl probe (5µM), 1µl human GAPDH primer/probe and 0.5µl nuclease-free water. The experiments were performed in triplicate for each reaction using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The data were analysed using the delta-delta Ct (ΔΔCt) method in the 7500 Software v2.0 (Applied Biosystems, USA) This method involves normalization of the sample cycle threshold (Ct) values against an endogenous control (ΔCt) and subsequently using this value relative to a selected

reference sample (corresponding to baseline value) to yield an absolute value of fold change. For analyses where reference samples were not applicable, normalized expression values were calculated using the following formula: $2^{-\Delta Ct}$.

Table 3.2: Primer and probe sequences for EBNA1, LMP1 and LMP2A genes

Gene	Sequences	
EBNA1	5' primer	GTGCGCTACCGGATGGC
	3' primer	CATGATTCACACTTAAAGGAGACGG
	Probe	TCCTCTGGAGCCTGACCTGTGATCG
LMP1	5' primer	AATTTGCACGGACAGGCATT
	3' primer	AAGGCCAAAAGCTGCCAGAT
	Probe	TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT
LMP2A	5' primer	CGGGATGACTCATCTCAACACATA
	3' primer	GGCGGTCACAACGGTACTAACT
	Probe	CAGTATGCCTGCCTGTAATTGTTGCGC

3.6.4 Plasmid Preparation

3.6.4.1 Bacterial transformation and propagation

Agar plates containing terrific broth (TB) (Fisher Scientific, USA), 1.5% agar (Fisher Scientific, USA) and 100μg/ml carbenicillin (Fisher Scientific, USA) were prepared and kept at 4^{o}C one day before the experiments. For bacterial transformation, DH5α competent *E. coli* cells were taken from -80^{o}C and thawed on ice. 300ng of the plasmid DNA was added to 40μl of the competent cells and the mixture was incubated on ice for 30 minutes. Cells were heat shocked by placing in a thermomixer (Eppendorf, Germany) at 42^{o}C for 45 seconds and returned to ice for 2 minutes. 260μl of TB was added to the cells and cultured in the thermomixer at 250rpm, 37^{o}C for 1 hour. 50μl of the transformed cells were plated on the agar plates and incubated overnight at 37^{o}C.

The next day, a single colony of the bacterial culture was picked and grown in TB containing 100μg/ml carbenicillin overnight on an orbital shaker at 37^{o}C.

3.6.4.2 Purification of plasmid DNA

Plasmid DNA was purified from the bacterial culture using a NucleoBond® Xtra Midi kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Briefly, bacterial cells were pelleted by centrifugation at 4500x g for 15 minutes and the supernatant was discarded. Cells were then lysed and the lysate was filtered through a NucleoBond® Xtra Column. The eluate was collected in a 15ml tube and room temperature isopropanol was added to the eluate. The plasmid DNA/isopropanol mixture was filtered through a NucleoBond® Finalizer and the precipitated plasmid DNA was bound to the filter membrane. The plasmid DNA was then washed with 70% ethanol, dried at room temperature for 5 minutes and eluted in 500μl of 5mM Tris/HCl buffer. Concentration of the plasmid DNA was measured using a NanoDrop 2000 spectrophotometer.

3.7 Western blotting

3.7.1 Protein extraction

Cells were grown in 100mm^2 dishes or 6-well plates until 80% confluence. Cells were washed with PBS, scraped and pelleted by centrifugation at 10,000rpm for 1 minute. Cells were then lysed in NP40 lysis buffer [150mM NaCl, 1% IGEPAL® CA-630 (Sigma-Aldrich, USA), 50mM Tris-HCl (pH8.0), Protease Inhibitor Cocktail Set III (Calbiochem, Germany) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, USA)] on ice for 30 minutes, followed by centrifugation at 13,200rpm, 4^{o}C for 30 minutes. The protein lysates (supernatant) were transferred to chilled 1.5ml tubes and stored at -80^{o}C.

3.7.2 Determination of protein concentration

Protein concentration was determined using a Bradford Protein Assay kit (Bio-rad, USA) according to the manufacturer's protocol. Bovine Serum Albumin (BSA) standard set (Bio-Rad, CA, USA), which consists of 7 different concentrations of BSA (0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2mg/ml), was used to construct a standard curve. The harvested protein lysate was diluted 1:10 with PBS. 250µl of 1x dye reagent was added to 5µl of diluted protein samples, PBS and BSA standards in a 96-well plate and incubated at room temperature for 5 minutes. The absorbance was measured at 595nm using a microplate reader. Protein concentration of the samples was determined from the standard linear curve plotted using the BSA standards.

3.7.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

50µg protein lysate was mixed with 2x Laemmli sample buffer (Bio-rad, USA) containing 5% β-mercaptoethanol (Bio-basic, Canada) and boiled at 70^{o}C for 10 minutes. Bio-rad apparatus was used for SDS-PAGE. 10% resolving gel was prepared and poured into the gel cassettes. Once the gel had set, 4% stacking gel was prepared and poured on top of the resolving gel with a gel comb inserted. The gel comb was removed after the gel had solidified. The gel was placed in the electrophoresis tank containing 1x running buffer (Thermo Scientific, USA). The denatured protein lysate and 5µl of Precision Plus Protein *All Blue* standard (Bio-rad, USA) were loaded into the wells. The proteins were separated in the stacking gel at 80V for 20 minutes, followed by 110V for 1 hour in the resolving gel.

3.7.4 Transferring and detection of protein

Following electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.45µm pore size; Merck Milipore, Germany). Prior to the transfer, the PVDF membrane was hydrated by soaking in

methanol for 15 seconds followed by 2 minutes in ultrapure water and 5 minutes in 1X TG Buffer (Bio-rad, USA) containing 20% methanol. Protein transfer was carried out using a Trans-Blot® Turbo Transfer System (Bio-Rad, CA, USA) at 25V for 30 minutes. The membrane was then blocked in 5% non-fat milk or 5% BSA (Bio-basic, Canada) in Tris-buffered Saline (TBS) [150mM NaCl, 50mM Tris-HCl (pH7.6)] with 0.1% Tween-20 (TBST) for 1 hour at room temperature followed by incubation with primary antibodies overnight at 4^{o}C. The following day, the PVDF membrane were washed three times for 5 minutes each in TBST prior to 1 hour incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000; Sigma-Aldrich, USA) or goat anti-mouse IgG (1:5000; Sigma-Aldrich, USA) diluted in 5% non-fat milk/TBST at room temperature. After further washes in TBST (3 x 5 minutes) and a 5-minute wash in TBS, the membrane was incubated with WesternBright Sirius enhanced chemiluminescene (ECL) reagent (Advansta, USA) and the target proteins were visualised using an Odyssey Fc Imaging System (LI-COR Biosciences, USA). The list of primary antibodies and the conditions used are listed in Table 3.3.

Table 3.3: List of primary antibodies for western blotting

Antibody	Species	Dilution	Blocking Buffer	Manufacturer
Anti-phospho-SPHK1 (Ser 225)	Rabbit polyclonal	1:1000	5% non-fat milk/TBST	ECM Biosciences, USA
Anti-total SPHK1	Rabbit polyclonal	1:1000	5% BSA/TBST	Cell Signaling Technology, USA
Anti-phospho-AKT (Ser473)	Rabbit polyclonal	1:1000	5% BSA/TBST	Cell Signaling Technology, USA
Anti-total AKT	Rabbit polyclonal	1:1000	5% BSA/TBST	Cell Signaling Technology, USA
Anti-phospho-p44/42 MAPK (ERK1/2)	Rabbit polyclonal	1:1000	5% BSA/TBST	Cell Signaling Technology, USA
Anti-total p44/42 MAPK (ERK1/2)	Rabbit polyclonal	1:1000	5% BSA/TBST	Cell Signaling Technology, USA
Anti-α-tubulin	Mouse monoclonal	1:10000	5% non-fat milk/TBST	Sigma-Aldrich, USA
Anti-β-actin	Mouse monoclonal	1:5000	5% non-fat milk/TBST	Sigma-Aldrich, USA

3.8 Immunofluorescence

Shandon multi-spot slides (Thermo Scientific, USA) were coated with 10μg/ml fibronectin overnight at 4^{o}C. Fibronectin was removed from the slide and 1.5 – 2x10^{4} cells were seeded onto each spot of the slides and allowed to adhere overnight at 37^{o}C. The following day, cells were washed with PBS gently and fixed with 100% ice-cold methanol for 15 minutes. The fixed cells were rinsed 3 times with PBS and blocked with 20% heat-inactivated normal goat serum (HINGS) diluted in PBS for 1 hour at room temperature. Primary antibodies [rabbit anti-EBNA1 (R4 rabbit serum; 1:1000; gift from Dr Christopher Dawson); mouse anti-LMP1 (1:50; Dako, Denmark); human anti-LMP2 (human NPC reference serum SK; 1:100; gift from Dr Christopher Dawson)] diluted in 20% HINGS/PBS were applied to the cells and incubated overnight

at 4^{o}C. Following 4 washes of 15 minutes each in PBS, the cells were incubated with AlexaFluor 488-conjugated goat anti-rabbit, anti-mouse or anti-human IgG (1:1000; Molecular Probes, USA) in 20% HINGS/PBS for 1 hour at room temperature. The cells were further washed in PBS (4 x 15 minutes) and mounted with 1,4-diazabicyclo[2.2.2]octane (DABCO) anti-fading agent for visualisation under a fluorescence microscope.

3.9 Statistical analysis

The data presented were representative of the experiments performed in triplicate. All the statistical analyses were carried out using GraphPad PRISM 5.0 software (GraphPad, USA). For Q-PCR analyses and *in vitro* assays, statistical differences between experimental groups were evaluated by Student's t-test or one-way analysis of variance (ANOVA)/Dunnett's test. Spearman's correlation was performed to examine the relationship between *SPHK1* and *S1PR3* in EBV-infected cells. p values <0.05 were considered as statistically significant.

CHAPTER 4:

THE PHENOTYPIC IMPACT OF EXOGENOUS S1P AND SPHK1 KNOCKDOWN ON NPC CELLS

4.1 Introduction

S1P is produced from sphingosine by sphingosine kinases (SPHK1 or SPHK2) and sphingosine is generated from ceramide by ceramidase. S1P is reversibly de-phosphorylated to sphingosine by S1P phosphatase or irreversibly converted to hexadecenal and ethanolamine phosphate by S1P lyase (Leong *et al.*, 2010). The balance between the levels of ceramide/sphingosine and S1P, termed sphingolipid rheostat, determines the cell fate. High levels of ceramide and sphingosine have been shown to exhibit anti-proliferative and pro-apoptotic effects in cancer cells (Pyne *et al.*, 2010). In contrast, the accumulation of S1P promotes cancer cell growth, survival, migration, invasion and angiogenesis (Takabe *et al.*, 2014).

The accumulation of S1P during carcinogenesis can often be attributed to the aberrant activation of SPHK1. SPHK1 is activated and then translocates to the plasma membrane through interaction with CIB1 to generate S1P (Jarman *et al.*, 2010). Accumulating evidence has shown that high expression of SPHK1 increased cell proliferation, survival and motility in various types of malignancies, such as cancers of colon, breast, kidney, ovary and lung (Shida *et al.*, 2008b). Overexpression of SPHK1 has recently been reported in NPC and was related to poor patient survival (Li *et al.*, 2015b). However, the functional roles of SPHK1 and S1P in the pathogenesis of NPC have not been studied.

The work in this chapter aimed to determine the phenotypic consequences of exogenous S1P and SPHK1 knockdown on NPC cells *in vitro*. The mechanisms associated with the phenotypic changes were also explored.

4.2 Effects of S1P on the phenotypes of NPC cells

To determine whether S1P affects the malignant phenotype of NPC cells, three different assays of cell behaviour were performed to examine cell proliferation, migration and invasion.

4.2.1 Cell proliferation

Four NPC cell lines, CNE1, HK1, HONE1 and TW04, were treated with a range of S1P concentrations (0.5, 1, 2.5, 5 and 10μM) and cell growth was measured by MTT assays. Since FBS contains S1P, the experiments were performed either in serum-free conditions or in media containing 0.1% or 0.5% FBS for 24 hours and 48 hours. Although there were some variations in the responses to S1P among the cell lines, in general, S1P inhibited the proliferation of all four cell lines examined irrespective of the serum concentrations (Figure 4.1).

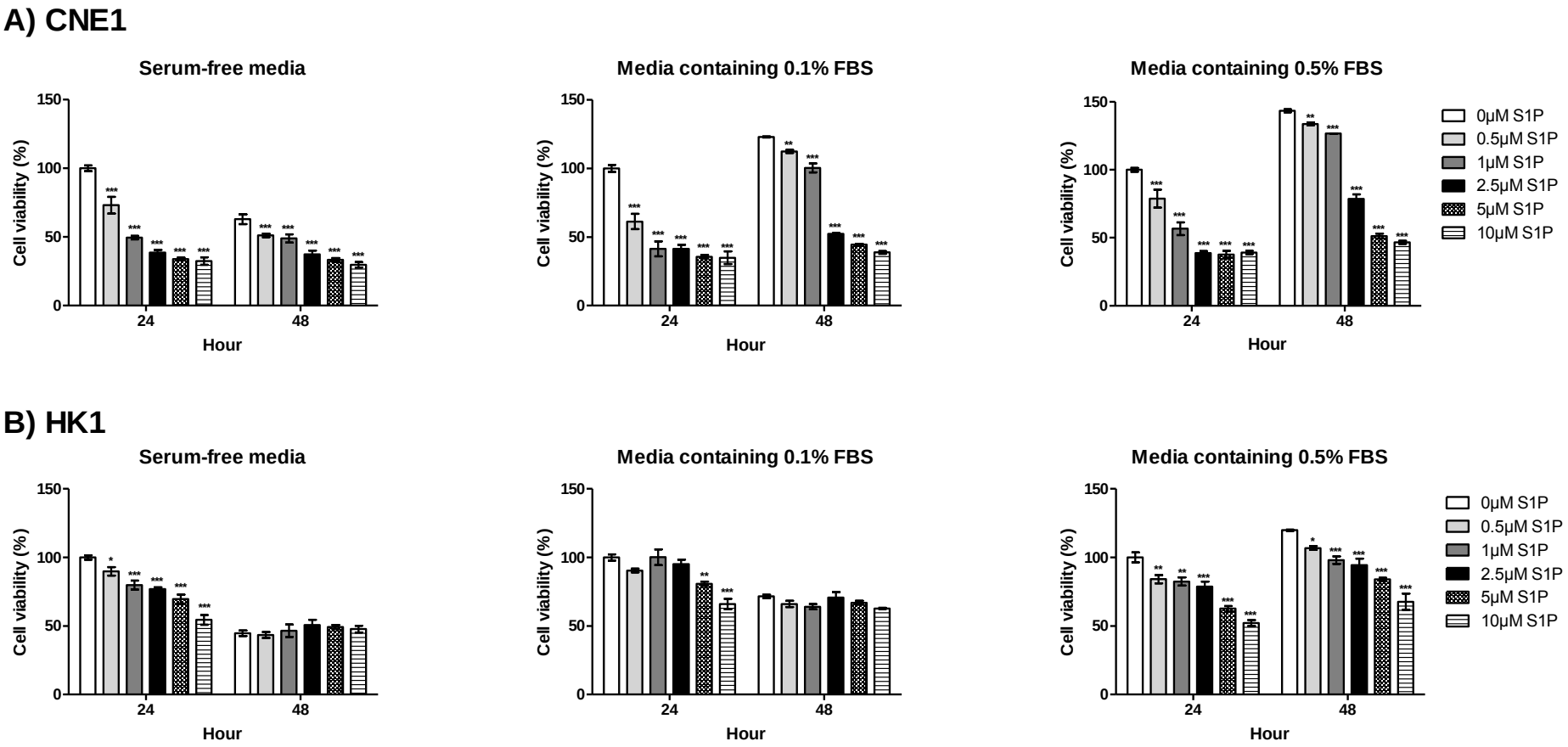
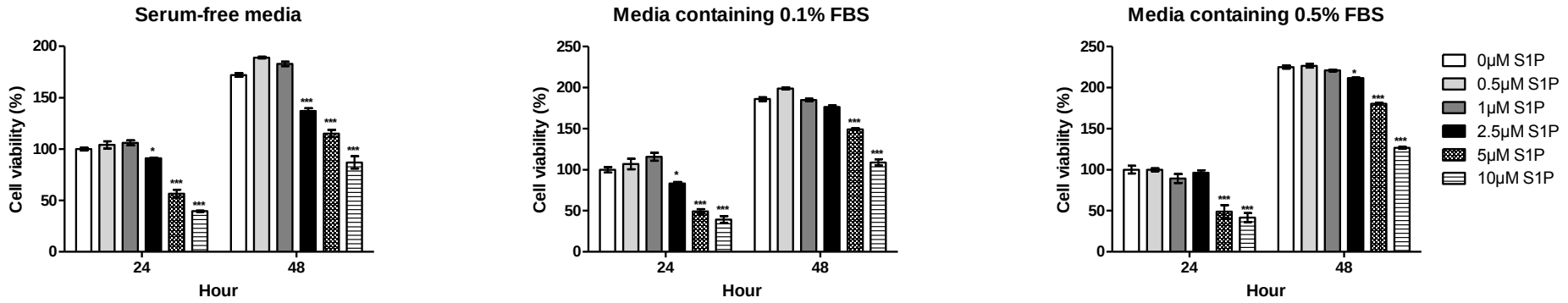


Figure 4.1: S1P inhibited the proliferation of NPC cells

The growth of (A) CNE1, (B) HK1, (C) HONE1 and (D) TW04 cells was examined in the absence or presence of FBS (0.1% or 0.5%) following S1P treatment at various concentrations (0, 0.5, 1, 2.5, 5 and 10μM) for 24 and 48 hours. In general, exogenous addition of S1P significantly reduced the growth of all four NPC cell lines. The data presented are representative of three independent experiments. *** denotes $p<0.001$, ** denotes $p<0.01$, * denotes $p<0.05$.

C) HONE1



D) TW04

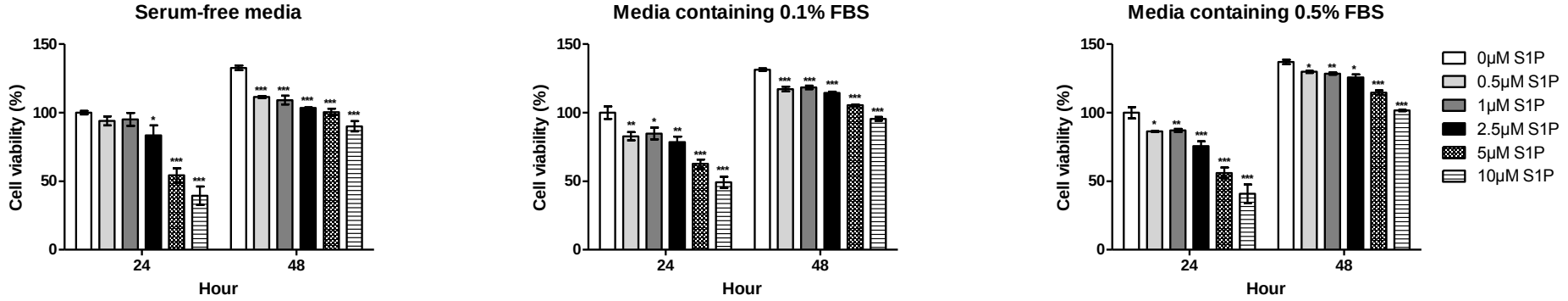


Figure 4.1, continued

4.2.2 Cell migration

Three NPC cell lines, TW04, HONE1 and SUNE1, were used to determine the effect of S1P on the migratory ability of NPC cells. Transwell migration assays with fibronectin-coated membranes were performed in the absence or presence of S1P (1μM or 5μM) in the lower chamber. Following addition of 1μM S1P, the migration of TW04, HONE1 and SUNE1 cells increased by 67%, 74% and 41%, respectively, compared to the controls. The migration of these cells was further increased by approximately 80% following addition of 5μM S1P ($p<0.001$; Figure 4.2). These results showed that S1P significantly promoted the migration of NPC cells *in vitro*.

4.2.3 Cell invasion

Given that cancer metastasis involves cell migration and invasion, transwell invasion assays were performed in a NPC cell line to determine whether S1P could also promote cell invasion *in vitro*. SUNE1 cells were treated with 5μM S1P and their invasive ability was measured using matrigel-coated Transwells. The results showed that S1P markedly enhanced the invasion of SUNE1 cells compared to the controls ($p<0.001$; Figure 4.3).

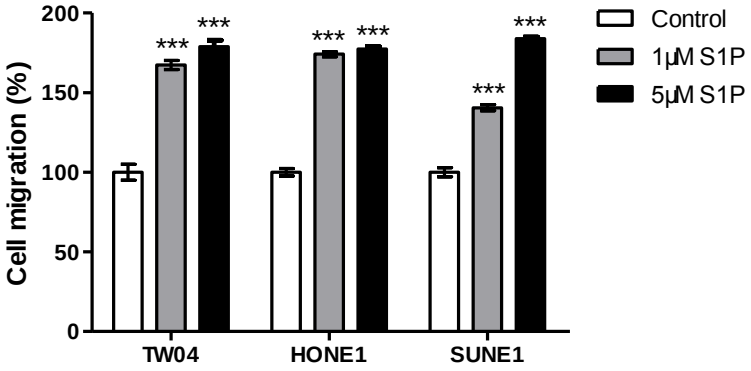


Figure 4.2: S1P promoted NPC cell migration

The migration of TW04, HONE1 and SUNE1 cells was examined using Transwell assays in the absence or presence of S1P (1 or 5μM). S1P significantly enhanced the migration of the three cell lines examined. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

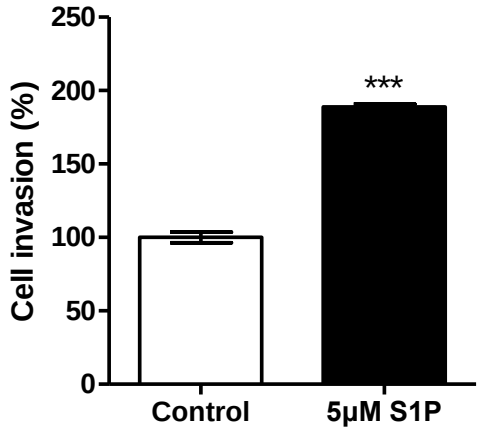


Figure 4.3: S1P increased NPC cell invasion

The invasive ability of SUNE1 cells was examined using matrigel-coated Transwell assays in the absence or presence of S1P (5μM). S1P markedly enhanced the invasion of SUNE1 cells. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

4.3 Biological significance of SPHK1 knockdown on NPC cell behaviour

Having shown that S1P influenced the phenotypes of NPC cells, I next examined whether knockdown of SPHK1, the key enzyme that produces S1P, would produce the opposite effects on cell proliferation and migration as the addition of exogenous S1P.

4.3.1 Validation of anti-SPHK1 antibodies

In order to determine the levels of SPHK1 protein in the NPC cells, two antibodies against SPHK1 were used: anti-phospho-SPHK1 antibody that only detects SPHK1 protein when it is phosphorylated at Ser225 and anti-total SPHK1 antibody that detects both the phosphorylated and non-phosphorylated forms of SPHK1 proteins. The specificity of the two antibodies was validated in HEK293 cells transfected with a vector that expressed the coding region of SPHK1 (pCMV6_XL4/SPHK1). Compared to the vector control (pCMV6_XL4), in western blots, a distinct band at 45kDa corresponding to the predicted molecular weight of SPHK1 protein was detected in HEK293 cells transfected with pCMV6_XL4/SPHK1, confirming that both antibodies were specific (Figure 4.4).

4.3.2 Expression of SPHK1 in NPC cell lines

To select cell lines for SPHK1 knockdown, I first compared the expression of SPHK1 in eight NPC cell lines with that in the immortalized nasopharyngeal cell line, NP69. The expression of *SPHK1* mRNA was significantly higher in three NPC cell lines (C666-1, HONE1 and TW01) ($p<0.001$; Figure 4.5), and an increase in the total SPHK1 protein was also observed in the two cell lines (C666-1 and HONE1) that expressed the highest levels of SPHK1 transcript ($p<0.05$; Figure 4.6).

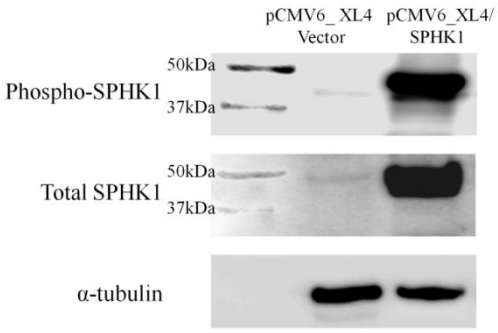


Figure 4.4: Validation of the specificity of antibodies against phosphorylated SPHK1 (Ser225) and total SPHK1 proteins

Western blot analyses of HEK293 cells transfected with pCMV6_XL4/SPHK1 showed a distinct band corresponding to the predicted molecular weight of SPHK1 (45kDa), confirming the specificity of the antibodies. Representative western blot images of two independent experiments are presented.

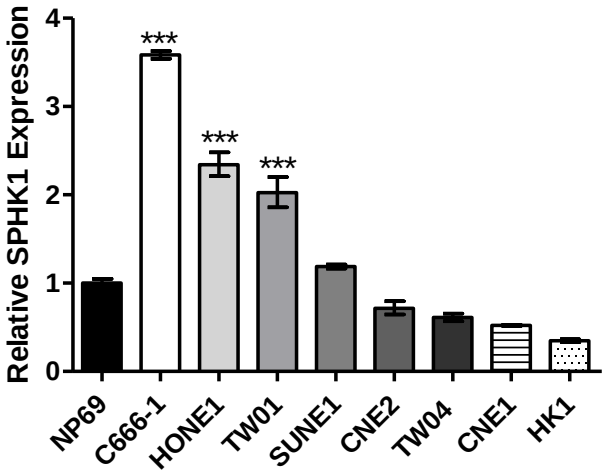


Figure 4.5: *SPHK1* mRNA expression in NPC cell lines

Q-PCR analyses showed that compared to the immortalised nasopharyngeal epithelial cell line NP69, three NPC cell lines (C666-1, HONE1 and TW01) expressed significantly higher levels of *SPHK1* mRNA. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

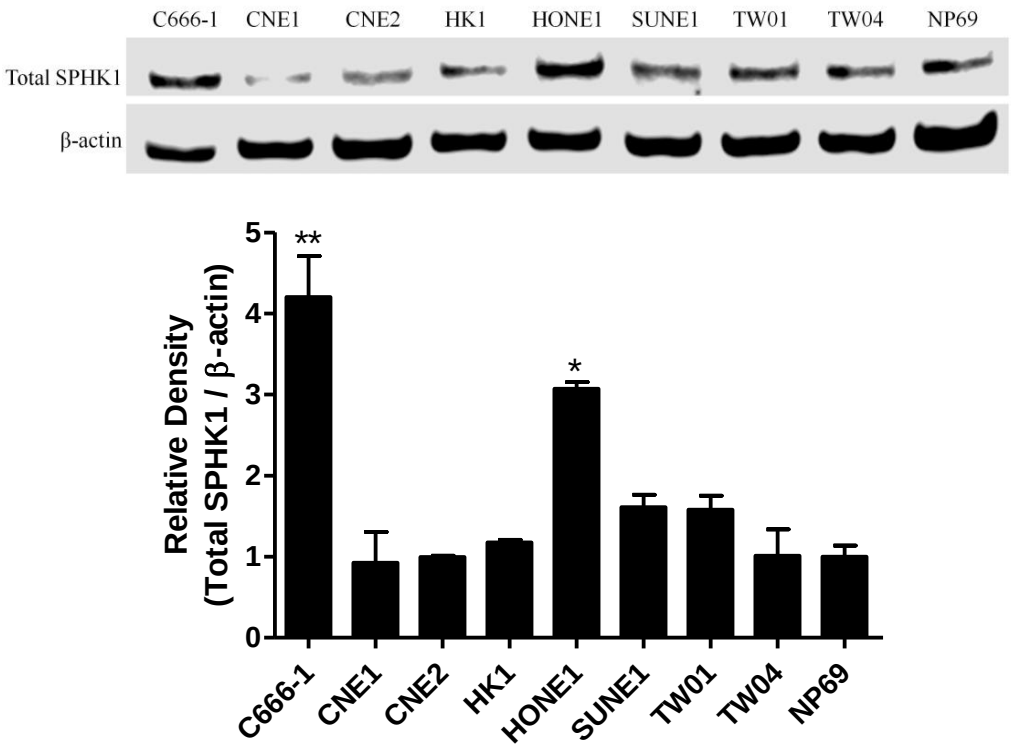


Figure 4.6: SPHK1 protein expression in NPC cell lines

Western blot analyses showed that C666-1 and HONE1 cells had higher protein levels of total SPHK1 compared to NP69. The densitometric data are expressed as the mean relative density (normalised to β-actin) ±SD from two independent experiments. ** denotes $p<0.01$, * denotes $p<0.05$.

4.3.3 Knockdown of SPHK1 in C666-1 and HONE1 cells

Two NPC cell lines, C666-1 and HONE1, were selected to perform the knockdown experiments based on their high levels of SPHK1. C666-1 and HONE1 cells were stably transduced with plasmids carrying two independent sequences of SPHK1 shRNAs (shSPHK1_S1 and shSPHK1_S2) or a non-targeting shRNA sequence (NS). Compared to NS, the *SPHK1* mRNA levels in C666-1/shSPHK1_S1 and C666-1/shSPHK1_S2 were reduced by approximately 40% whereas the *SPHK1* mRNA levels in HONE1/shSPHK1_S1 and HONE1/shSPHK1_S2 were reduced by 62% and 93%, respectively ($p<0.001$; Figure 4.7). In agreement with these data, knockdown of SPHK1 which would reduce the levels of endogenous S1P, also led to a decrease in both the phosphorylated and total SPHK1 proteins (Figure 4.8). The knockdown also appeared to be specific for SPHK1 with no noticeable effect on α-tubulin levels. These results showed that shSPHK1_S2 produced a more efficient knockdown compared to shSPHK1_S1 in both cell lines and based on the mRNA levels, the knockdown of SPHK1 appeared to be more effective in HONE1 cells. Given that C666-1 cells do not exhibit the ability to migrate in response to S1P under serum-free conditions, only HONE1 cells were used for the subsequent experiments.

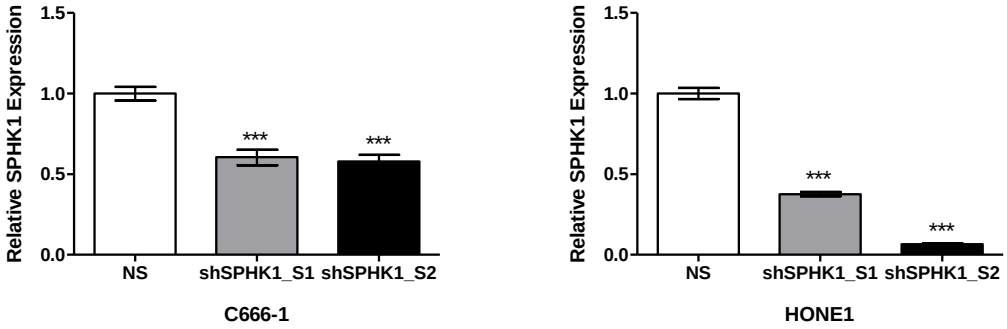


Figure 4.7: *SPHK1* mRNA expression in C666-1 and HONE1 cells following knockdown of SPHK1

C666-1 and HONE1 cells were stably transduced with plasmids carrying two independent sequences of SPHK1 shRNAs (shSPHK1_S1 and shSPHK1_S2) or the non-targeting shRNA sequence (NS). Compared to NS, knockdown of SPHK1 in C666-1 and HONE1 cells resulted in approximately 40% and 62%-93% reduction of SPHK1, respectively. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

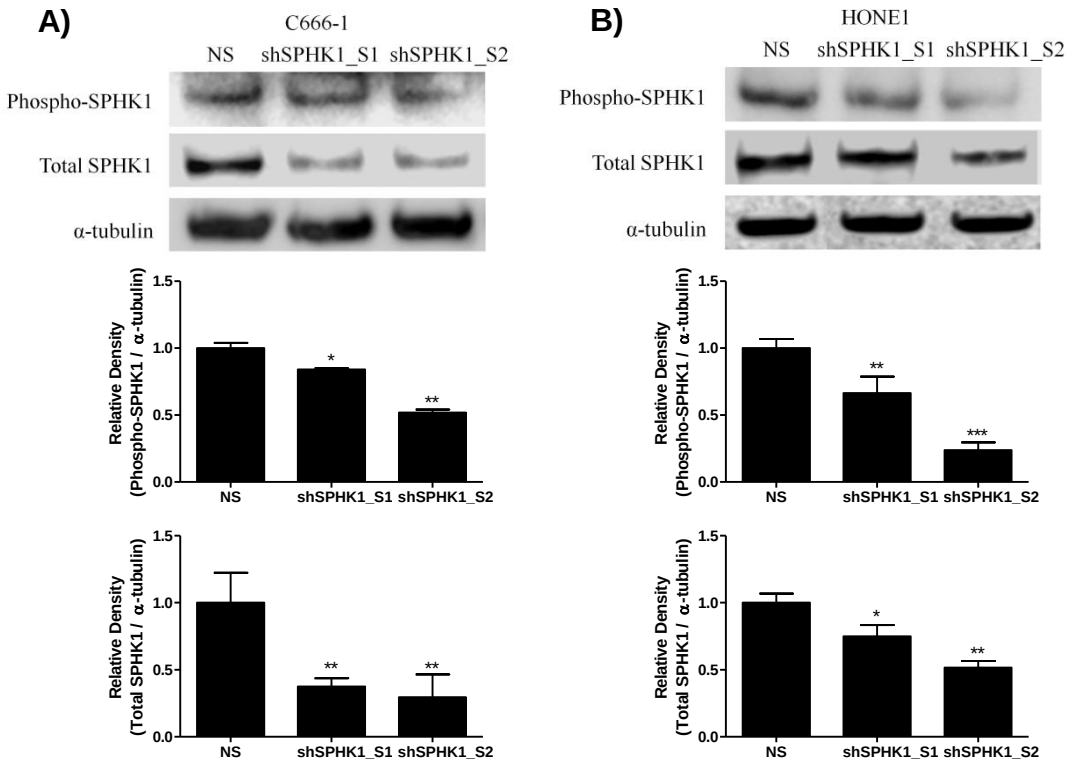


Figure 4.8: SPHK1 protein levels following SPHK1 knockdown in C666-1 and HONE1 cells

Western blot analyses showed that the levels of both phospho-SPHK1 and total SPHK1 proteins were decreased in (A) C666-1 and (B) HONE1 cells following SPHK1 knockdown. The densitometric data are expressed as the mean relative density (normalised to α-tubulin) ±SD from three independent experiments. *** denotes $p<0.001$, ** denotes $p<0.01$, * denotes $p<0.05$.

4.3.4 Effect of SPHK1 knockdown on cell proliferation

The consequence of SPHK1 knockdown on the growth of HONE1 cells was determined using MTT assays over a period of 5 days. Compared to the controls, knockdown of SPHK1 significantly inhibited the growth of HONE1 cells from the third day onwards ($p<0.001$; Figure 4.9). As knockdown of SPHK1 would likely result in reduced levels of S1P, these data were unexpected because exogenous addition of S1P was previously shown to suppress the growth of NPC cells, including HONE1 cells (Figure 4.1). These contradictory results are discussed in chapter 7.

4.3.5 Effect of SPHK1 knockdown on cell migration

Transwell migration assays were performed to determine the migratory ability of HONE1 cells following SPHK1 knockdown. Compared to HONE1/NS cells, SPHK1 knockdown significantly suppressed the migration of HONE1/shSPHK1_S1 and HONE1/shSPHK1_S2 cells by 36% and 61%, respectively ($p<0.001$; Figure 4.10). These data were in agreement with the results obtained previously that addition of S1P enhanced the migration of NPC cells (Figure 4.2).

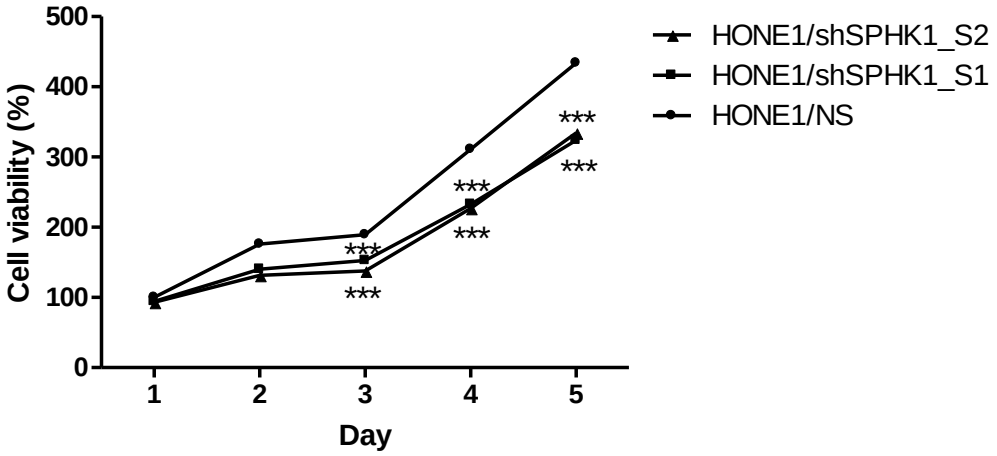


Figure 4.9: Knockdown of SPHK1 decreased NPC cell proliferation

Following SPHK1 knockdown, HONE1 cells grew slower compared to the controls. The data presented are representative of two independent experiments (error bars are too small to be visible). *** denotes $p<0.001$.

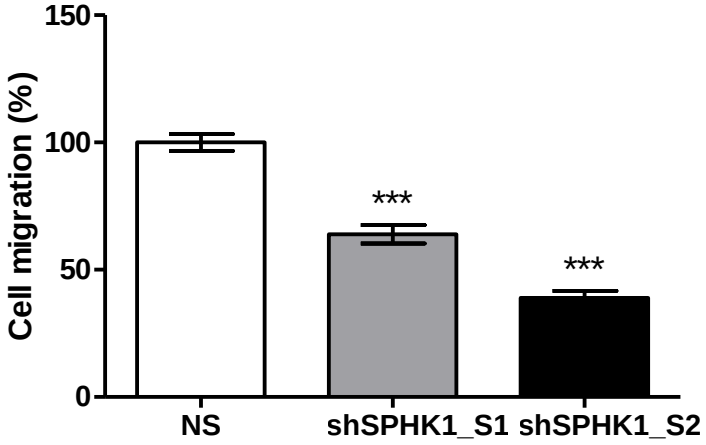


Figure 4.10: Knockdown of SPHK1 inhibited NPC cell migration

The effect of SPHK1 knockdown on the migration of NPC cells was examined using Transwell assays in the presence of 10% FBS in the lower chambers. Compared to HONE1/NS, knockdown of SPHK1 significantly inhibited the migration of HONE1 cells. The data presented are representative of three independent experiments. *** denotes $p<0.001$.

4.4 Activation of AKT and ERK pathways by S1P

Next, I investigated the downstream signalling pathways that were activated by S1P in NPC cells. AKT and ERK signalling pathways were chosen because they represent two main downstream targets of S1P signalling (Pyne *et al.*, 2010) and are also frequently activated in NPC (Tulalamba *et al.*, 2012). The activation of AKT and ERK signalling was determined by measuring the levels of phosphorylated AKT (phospho-AKT) and phosphorylated ERK (phospho-ERK), respectively.

Following exogenous addition of S1P, the levels of phospho-AKT and phospho-ERK proteins were examined in two NPC cell lines, HONE1 and TW04. Western blot analyses showed that the levels of phospho-AKT were increased in HONE1 and TW04 cells following S1P treatment (Figure 4.11). The phosphorylation of ERK was also found to be stimulated in HONE1 and TW04 cells by S1P treatment (Figure 4.12). These results suggested that both the AKT and ERK signalling pathways were activated in response to exogenous S1P in NPC cells. The levels of both total AKT and total ERK proteins in these two cell lines were unaffected by S1P treatment, implying that S1P did not modulate the translation process of AKT and ERK.

To further confirm that the AKT and ERK pathways are targets of the S1P signalling cascade, the levels of phospho-AKT and phospho-ERK were determined in HONE1 cells following SPHK1 knockdown. Western blot analyses demonstrated that knockdown of SPHK1 inhibited the phosphorylation of AKT in HONE1 cells, but not the levels of total AKT protein ($p<0.05$; Figure 4.13). In contrast, the levels of phospho-ERK and total ERK proteins remained unchanged in these cells (Figure 4.14). Given that knockdown of endogenous SPHK1 appeared to only suppress the activation of AKT, subsequent experiments were focused on the involvement of AKT signalling in S1P-mediated migration of NPC cells.

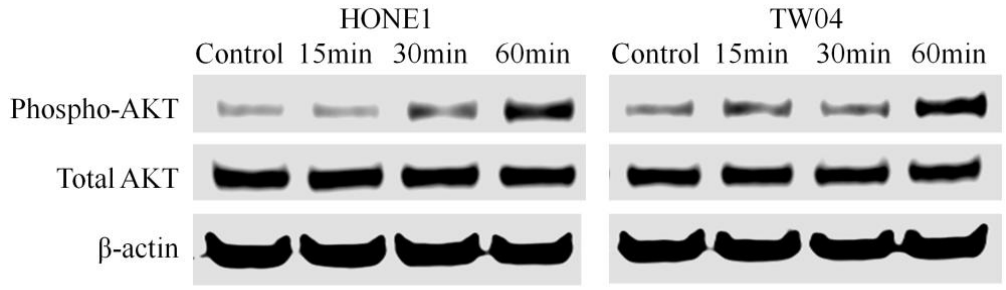


Figure 4.11: S1P activated AKT signalling in NPC cells

Compared to the untreated controls, the levels of phospho-AKT were increased in HONE1 cells at 30 minutes following treatment with 5μM S1P and remained activated at 60 minutes whereas the phosphorylation of AKT in TW04 cells was only observed at 60 minutes post-S1P treatment. Representative western blot images of three independent experiments are presented.

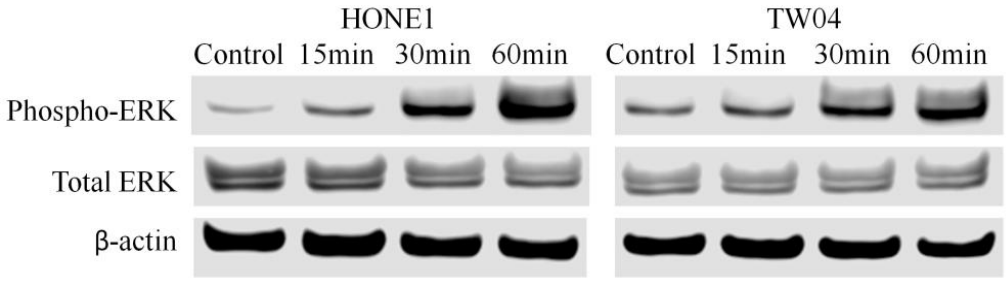


Figure 4.12: S1P activated ERK signalling in NPC cells

HONE1 and TW04 cells showed increased levels of phospho-ERK 15 minutes and 30 minutes following exogenous addition of 5μM S1P, respectively, compared to the untreated controls. The activation of ERK remained in both cell lines at 60 minutes post-S1P treatment. Representative western blot images of three independent experiments are presented.

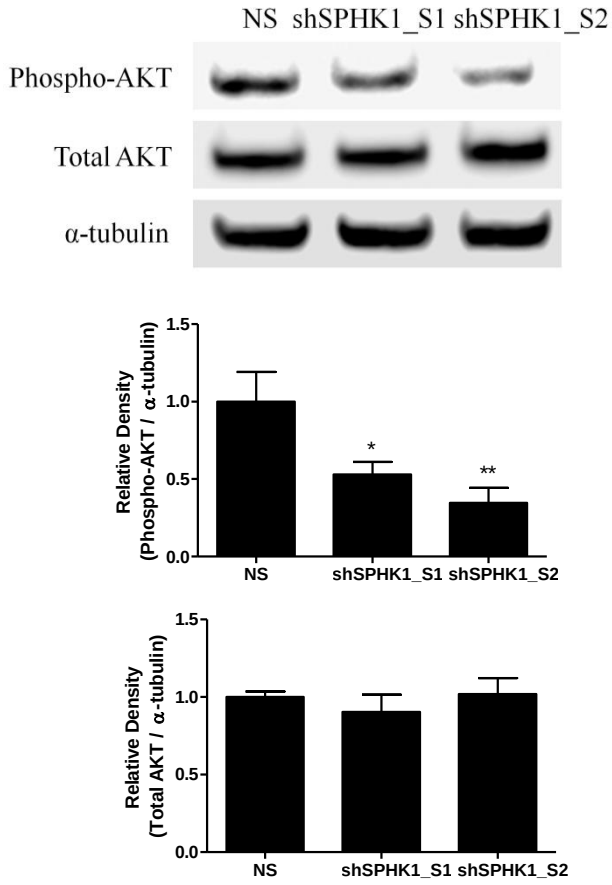


Figure 4.13: Knockdown of SPHK1 in HONE1 cells suppressed the activation of AKT

Compared to the HONE1/NS, knockdown of SPHK1 reduced the levels of phospho-AKT, but not the total AKT proteins in HONE1 cells. The densitometric data are expressed as the mean relative density (normalised to α-tubulin) ±SD from three independent experiments ** denotes $p<0.01$, * denotes $p<0.05$.

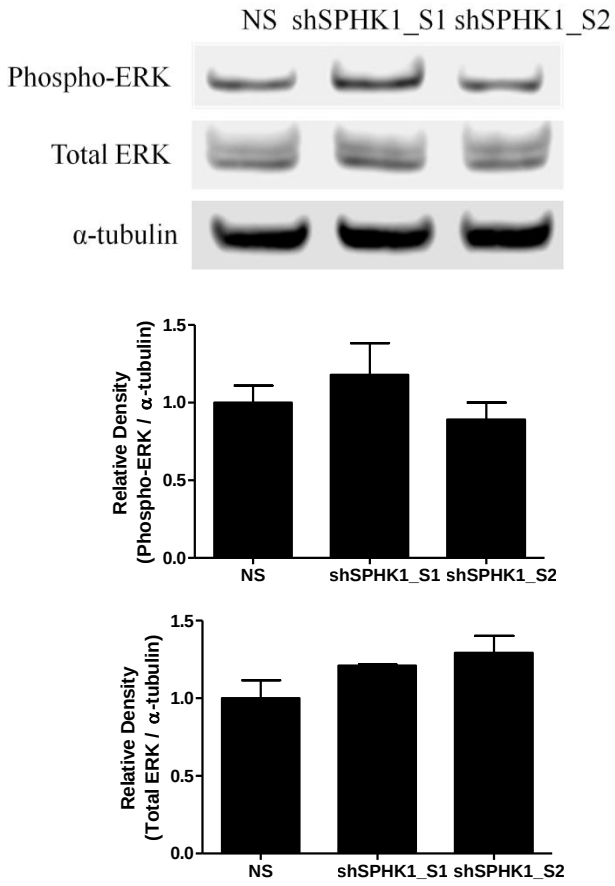


Figure 4.14: Knockdown of SPHK1 in HONE1 cells did not affect the expression and activation of ERK

Both the phosphorylated and total ERK proteins in HONE1 cells remained unchanged following knockdown of SPHK1. The densitometric data are expressed as the mean relative density (normalised to α-tubulin) ±SD from three independent experiments.

4.5 Involvement of AKT signalling in S1P-induced migration

4.5.1 Establishment of LY294002 kill curves

To investigate whether S1P induced NPC cell migration through the activation of AKT, HONE1 and SUNE1 cells were treated with a PI3K/AKT inhibitor, LY294002. Dose response experiments (LY294002, 0.5 – 10μM) in HONE1 and SUNE1 cells showed that 1μM LY294002 did not affect cell viability (Figure 4.15) but significantly reduced phospho-AKT levels in SUNE1 cells (Figure 4.16).

4.5.2 Effect of LY294002 treatment on S1P-induced migration

As shown in Figure 4.17, addition of S1P markedly increased the migration of HONE1 and SUNE1 cells. Treatment with LY294002 alone had no effect on the migration of both cell lines. In the presence of S1P, the migration of HONE1 and SUNE1 cells was reduced by approximately 50% compared to their respective controls following LY294002 treatment. These data suggested the involvement of AKT signalling in S1P-induced migration.

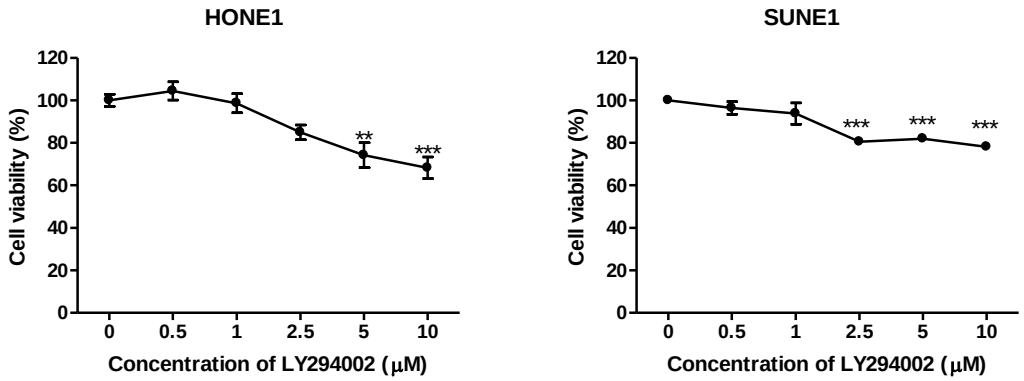


Figure 4.15: NPC cell viability following LY294002 treatment

MTT cell viability assays were used to assess the viability of cells following treatment with LY294002 (0 - 10μM). Concentrations of LY294002 above 1μM were cytotoxic and reduced cell viability. The data presented are representative of two independent experiments. *** denotes $p<0.001$, ** denotes $p<0.01$.

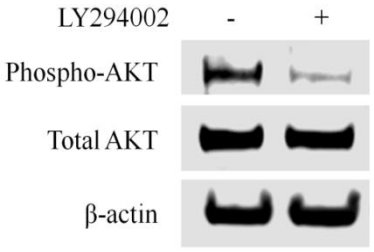


Figure 4.16: LY294002 treatment in SUNE1 cells inhibited the activation of AKT

Western blot analyses demonstrated that treatment with 1μM LY294002 decreased the levels of phospho-AKT in SUNE1 cells. Representative western blot images of two independent experiments are shown.

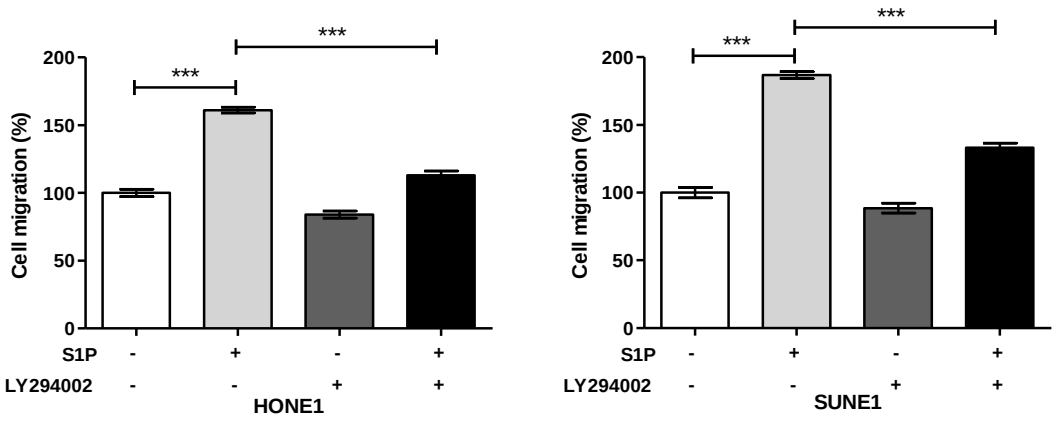


Figure 4.17: Inhibition of AKT suppressed S1P-induced NPC cell migration

The migration of HONE1 and SUNE1 cells were examined using Transwell assays in the absence or presence of S1P (5μM) and/or LY294002 (1μM). In the presence of S1P, the migration of both cell lines was significantly inhibited following treatment with LY294002. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

4.5.3 Expression of constitutively active AKT reverses the anti-migratory effects of SPHK1 knockdown in NPC cells

Having shown that knockdown of SPHK1 inhibited the activation of AKT in HONE1 cells, rescue experiments were carried out to determine whether expression of a constitutively active AKT could reverse the suppressive effect of SPHK1 knockdown on cell migration. Western blot analyses confirmed the expression of phospho-AKT in HONE1 following the transfection of a constitutively active AKT (Figure 4.18). While SPHK1 knockdown significantly suppressed the migration of HONE1 cells, the expression of constitutively active AKT led to an increase in the migration of SPHK1-knockdown cells by approximately 20% ($p<0.001$; Figure 4.19). These results supported previous observations that S1P induced the migration of NPC cells through the activation of AKT (Figure 4.17).

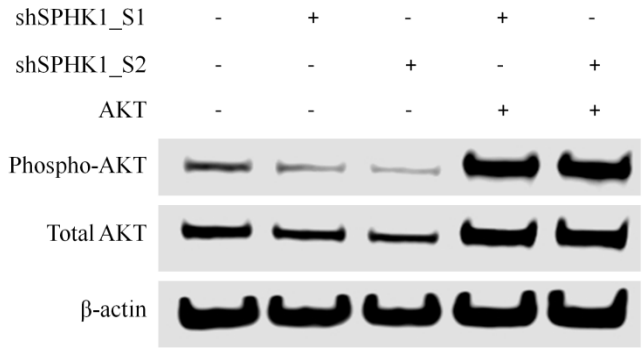


Figure 4.18: Expression of phospho-AKT protein following transfection of a constitutively active AKT

Western blot analyses confirmed the expression of phospho-AKT in SPHK1-knockdown HONE1 cells following the transfection of a constitutively active AKT construct. Representative western blot images of two independent experiments are presented.

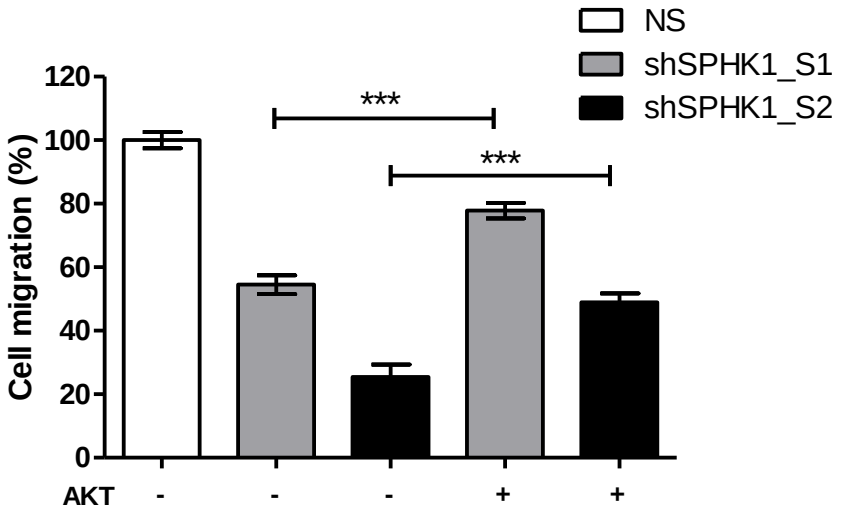


Figure 4.19: Expression of constitutively active AKT rescued the suppressive effect of SPHK1 knockdown on HONE1 cell migration

SPHK1-knockdown HONE1 cells were transfected with a constitutively active AKT and the migratory ability was determined using Transwell assays in the presence of 10% FBS. Expression of a constitutively active AKT restored the migration of SPHK1-knockdown HONE1 cells. The data presented are representative of two independent experiments. *** denotes $p<0.001$.

4.6 Summary

In this chapter, the effects of S1P on the behaviour of NPC cells were investigated using assays of cell proliferation, migration and invasion. Although exogenous S1P reduced NPC cell proliferation, it significantly promoted the migration and invasion of NPC cells.

High expression of SPHK1 in tumour cells is predicted to result in elevated levels of S1P. Therefore, to determine whether endogenous SPHK1 has a similar effect on cell migration and proliferation as the addition of S1P, knockdown of SPHK1 in C666-1 and HONE1 cells was performed using two shRNA sequences. Since the knockdown was more effective in HONE1 cells and they retained the ability to migrate in response to S1P under serum-free conditions, only this cell line was used in the subsequent experiments. In agreement with the previous results that S1P enhanced NPC cell migration, knockdown of SPHK1 resulted in decreased migration of HONE1 cells. However, while treatment of cells with S1P reduced cell proliferation, knockdown of SPHK1 also inhibited the growth of HONE1 cells.

Next, the downstream targets of S1P signalling in NPC cells were examined. Western blot analyses showed that S1P induced the phosphorylation of AKT and ERK in HONE1 and TW04 cells. However, knockdown of SPHK1 in HONE1 cells reduced only the phosphorylated levels of AKT, but not ERK. The involvement of AKT signalling in S1P-induced migration was further explored using the PI3K/AKT inhibitor, LY294002. Treatment of HONE1 and SUNE1 cells with LY294002 significantly suppressed S1P-induced migration. In agreement with these data, expression of a constitutively active AKT was able to rescue the suppressive effect of SPHK1 knockdown on HONE1 cell migration. Collectively, these results demonstrate that S1P induced the migration of NPC cells via the activation of AKT.

CHAPTER 5:

IDENTIFICATION OF THE S1P RECEPTORS THAT MEDIATE S1P-INDUCED MIGRATION IN NPC

5.1 Introduction

S1P is produced in the cytoplasm and transported out from the cells by various transporter proteins, including ABCA1, ABCC1, ABCG2 and SPNS2 (Takabe *et al.*, 2014). Extracellular S1P binds to one or more of its five S1P receptors, S1PR1-5, in a paracrine or autocrine manner, to trigger several signalling pathways such as PI3K/AKT, ERK and JNK (Rosen *et al.,* 2009; Takabe *et al.,* 2008). This process is referred to as "inside-out" signalling (Spiegel *et al.,* 2011).

The oncogenic effects of S1P have been suggested to be a consequence of alterations in the expression or function of the S1P receptors (Blaho *et al.*, 2014). S1PR1 and S1PR3 promote cancer progression by increasing cell growth, survival, angiogenesis, migration, invasion and chemotherapeutic drug resistance (Pyne *et al.,* 2012; Watters *et al.*, 2011). S1PR2 is generally considered as a tumour suppressor that inhibits cell proliferation, motility and angiogenesis (Takuwa *et al.*, 2011), but accumulating evidence also shows that it can exhibit tumour-promoting effects by increasing cell growth, survival, migration and invasion (Adada *et al.,* 2013; Patmanathan *et al.,* 2016). The functional properties of S1PR4 and S1PR5 in cancer development remain largely unclear.

In this chapter, I identified which S1PR(s) were responsible for the S1P-mediated NPC cell migration described in the previous chapter.

5.2 Expression of S1PRs in NPC primary tissues and cell lines

Two published microarray datasets, GSE12452 (Sengupta *et al.,* 2006) and GSE34573 (Hu *et al.,* 2012) that compared the expression profiles of micro-dissected

NPC tissues and non-malignant nasopharyngeal epithelium were re-analysed by Dr Wenbin Wei (University of Birmingham, UK) to determine the expression of five S1PRs in NPC primary tissues. The GSE12452 dataset consists of 31 NPC samples and 10 non-malignant controls, whereas the GSE34572 dataset comprises of 15 NPC samples and 3 non-malignant controls. The analyses showed that only *S1PR3* was significantly and consistently overexpressed in NPC in both datasets, with an increase of 1.7- and 4-fold in GSE12452 and GSE34573, respectively ($p<0.05$, Figure 5.1). Upregulation of *S1PR2* and *S1PR5* in NPC was only evident in GSE12452 with a fold change of 1.2 ($p<0.05$) and 1.7 ($p<0.01$), respectively.

Q-PCR analyses were used to determine the expression of all five S1PRs in a panel of NPC cell lines. The results showed that the expression of *S1PR2* and *S1PR5* were readily detected in all the eight NPC cell lines examined while the levels of *S1PR3* varied (Figure 5.2). Although there were some variations in the expression of *S1PR1* and *S1PR4*, NPC cell lines generally expressed low levels of *S1PR1* and *S1PR4* compared to other S1PRs. Given that the reagents to study S1PR5 are limited, S1PR2 and S1PR3 were selected as the candidate receptors for subsequent experiments.

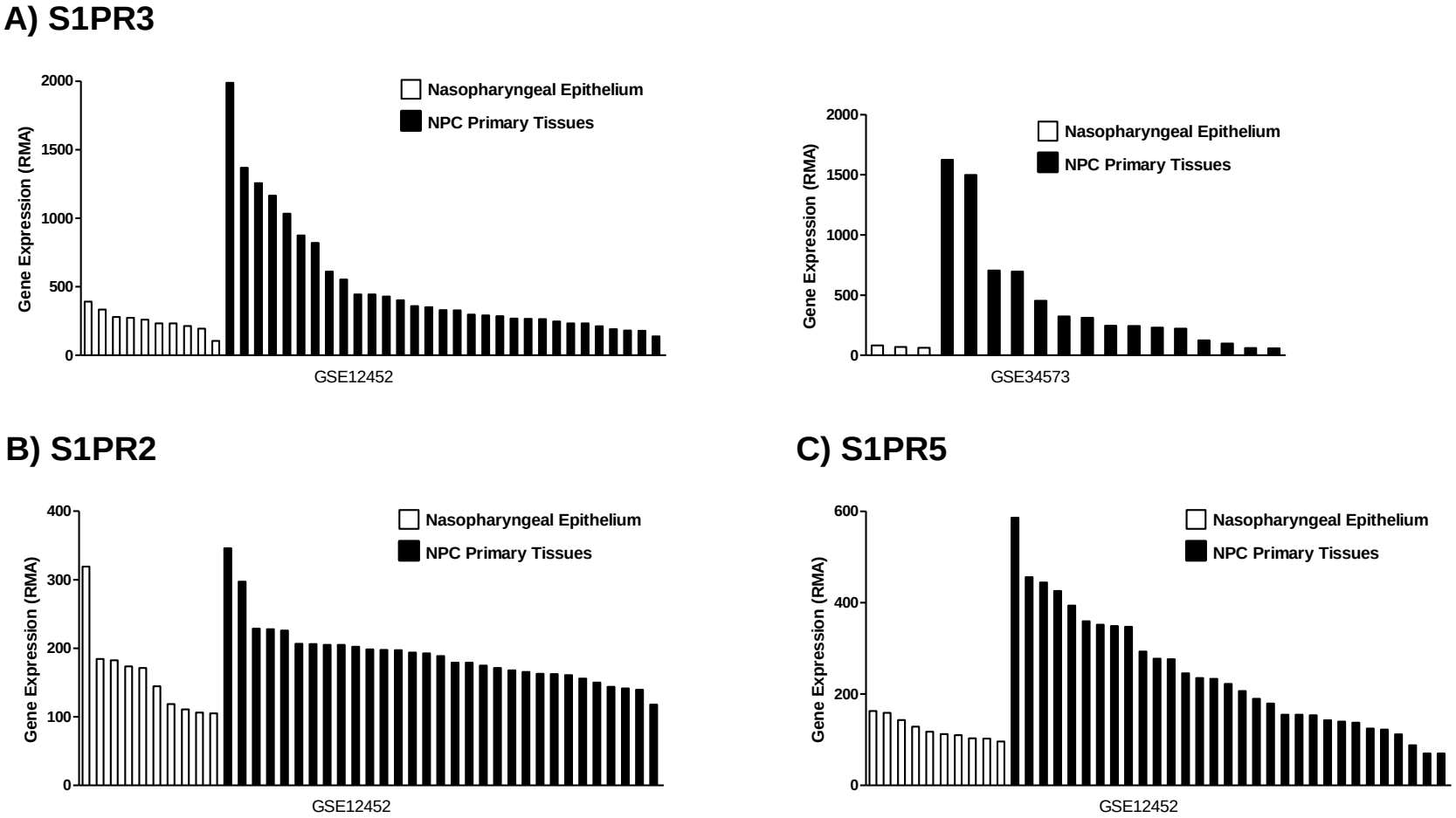


Figure 5.1: Expression of S1PR2, S1PR3 and S1PR5 in NPC primary tissues

Re-analyses of the GSE12452 and GSE34572 microarray datasets showed that *S1PR3* (A) was over-expressed in micro-dissected primary NPC tissues compared to nasopharyngeal epithelium in both datasets, whereas overexpression of *S1PR2* (B) and *S1PR5* (C) was only evident in the GSE12452 dataset.

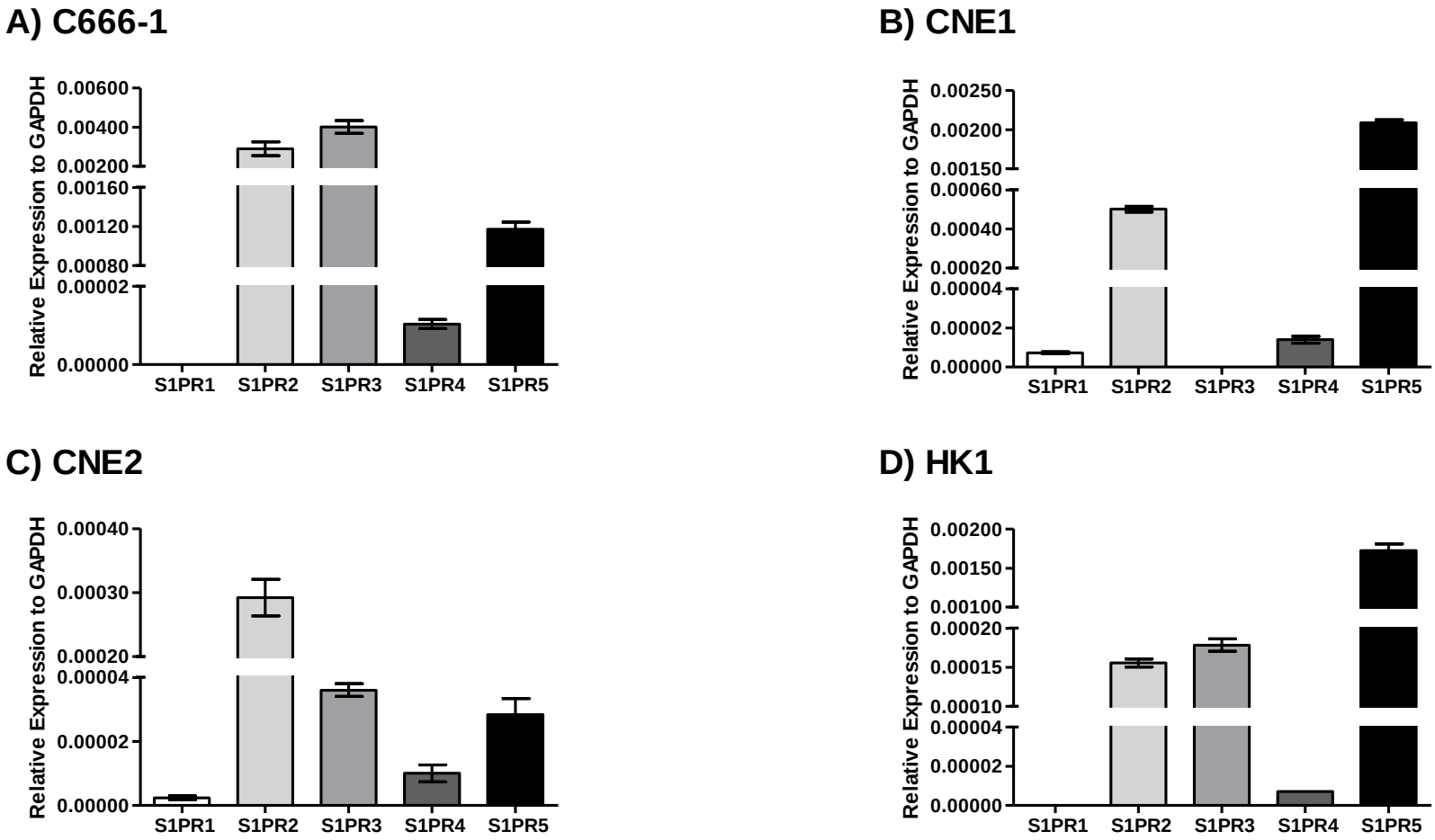


Figure 5.2: Expression profile of S1PRs in NPC cell lines

Q-PCR analyses showed that the expression of *S1PR2* and *S1PR5* were readily detected in all eight NPC cell lines, (A) C666-1, (B) CNE1, (C) CNE2, (D) HK1, (E) HONE1, (F) SUNE1, (G) TW01 and (H) TW04 while the expression of *S1PR3* varied and the levels of S1PR1 and S1PR4 were generally low. The data presented are representative of two independent experiments.

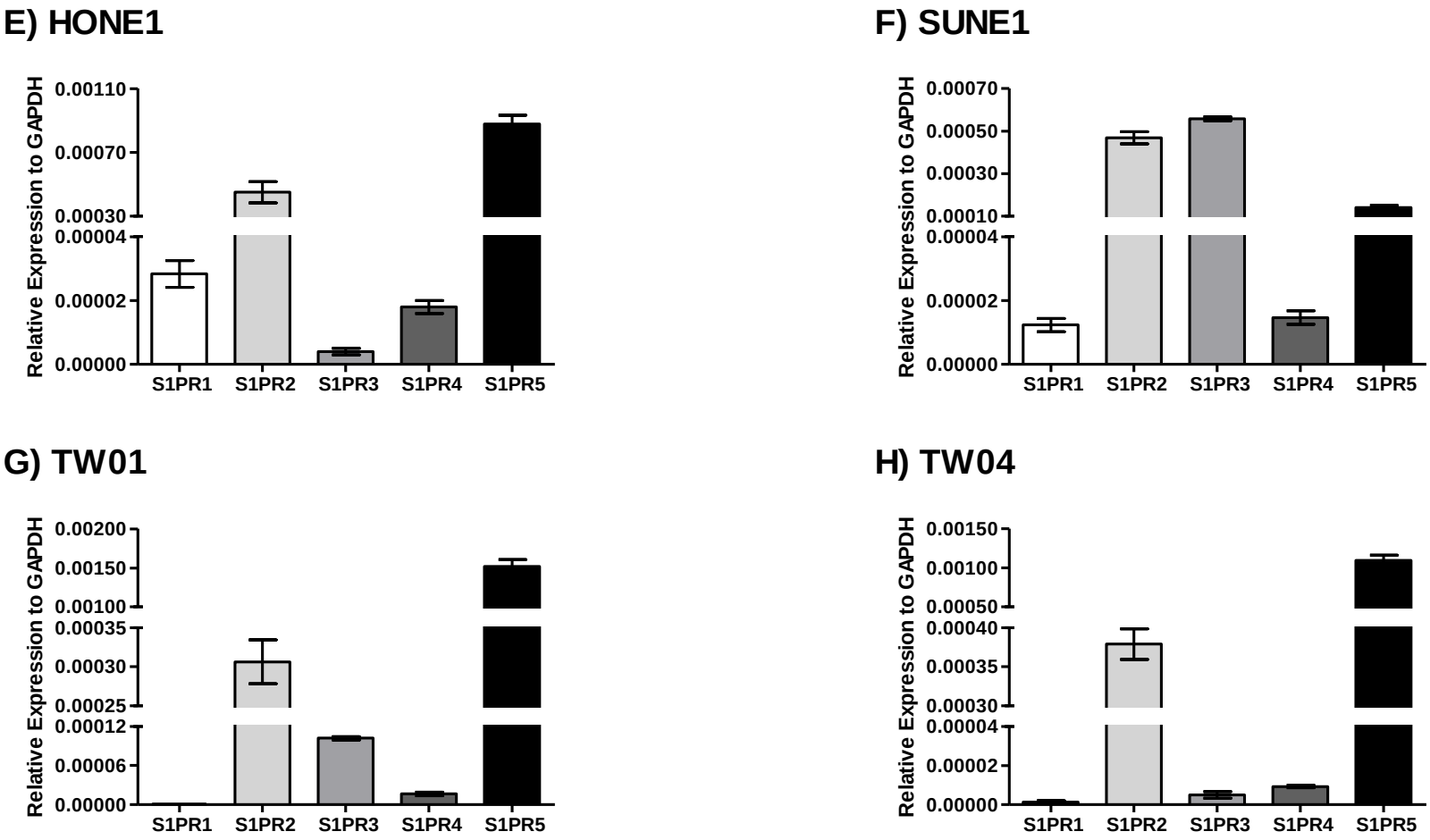


Figure 5.2, continued

5.3 Involvement of S1PR2 and S1PR3 in S1P-induced migration

To determine the roles of S1PR2 and S1PR3 in S1P-mediated migration, several pharmacological reagents targeting these receptors were used.

5.3.1 JTE-013

JTE-013 is a S1PR2/S1PR4 antagonist that inhibits the binding of S1P to S1PR2 and/or S1PR4 (Long *et al.*, 2010b; Ohmori *et al.*, 2003). Kill curves of JTE-013 in HONE1 and SUNE1 cells were generated using MTT cell viability assays following treatment with JTE-013 (0.1 – 10μM) for 24 hours. The results showed JTE-013 was not cytotoxic at any of the concentrations tested (Figure 5.3); 1μM JTE-013 was selected for subsequent migration assays because this is the most commonly used concentration in published studies (Salomone *et al.*, 2011).

The migratory ability of HONE1 and SUNE1 cells following JTE-013 treatment was examined using Transwell migration assays with fibronectin-coated membranes. Compared to the vehicle controls, addition of JTE-013 alone did not affect the migration of either cell line (Figure 5.4). However, in the presence of S1P, treatment with JTE-013 significantly inhibited the migration of HONE1 and SUNE1 cells by 16% and 39%, respectively ($p<0.001$). These results suggested that S1PR2 might play a role in S1P-induced migration of NPC cells.

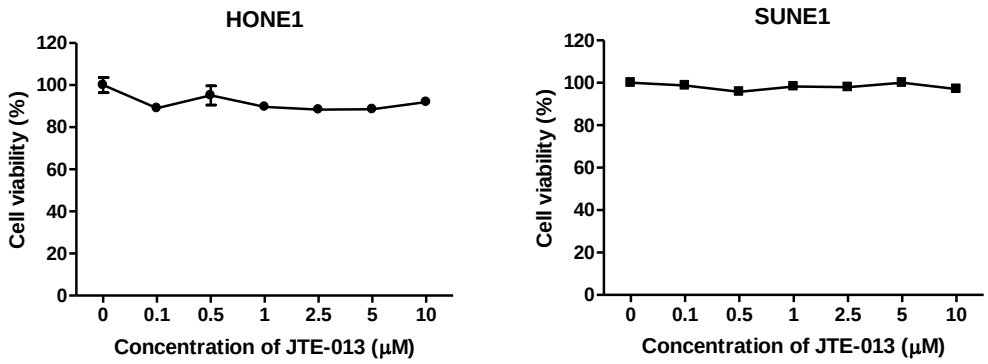


Figure 5.3: NPC cell viability following treatment with JTE-013 (a S1PR2/S1PR4 antagonist)

Viability of HONE1 and SUNE1 cells following JTE-013 treatment was determined by MTT assays. Treatment with up to 10μM JTE-013 did not affect the viability of either cell line. The data presented are representative of two independent experiments (error bars for most of the concentrations are too small to be visible).

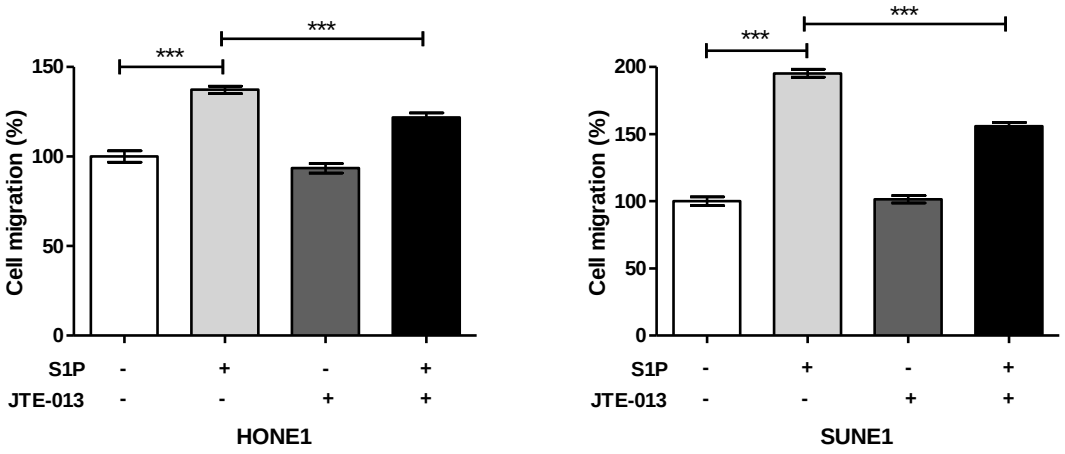


Figure 5.4: Inhibition of S1PR2 suppressed S1P-induced migration of NPC cells

The migration of HONE1 and SUNE1 cells was examined using Transwell assays in the absence or presence of S1P (5μM) and/or JTE-013 (1μM). Similar to the previous results (Figure 4.2), S1P increased the migration of HONE1 and SUNE1 cells and these effects were significantly suppressed by JTE-013. The data presented are representative of two independent experiments. *** denotes $p<0.0001$.

5.3.2 CYM-5478

Having shown that JTE-013 inhibited S1P-induced migration of HONE1 and SUNE1 cells, the role of S1PR2 on NPC cell migration was further explored using a S1PR2 allosteric agonist, CYM-5478. CYM-5478 does not compete with JTE-013 for binding to S1PR2 and the responses triggered by CYM-5478 can be inhibited by JTE-013 (Herr *et al.*, 2016; Satsu *et al.*, 2013). Since HONE1 and SUNE1 cells expressed similar levels of S1PR2 (Figure 5.2), only SUNE1 cells were used in these experiments. SUNE1 cells were treated with CYM-5478 (1 – 10μM) for 24 hours and none of these concentrations was shown to be cytotoxic to the cells (Figure 5.5). Therefore, 10μM CYM-5478 was chosen for subsequent experiments as this concentration has previously been shown to activate S1PR2 effectively in cell-based assays (Herr *et al.*, 2016).

While S1P significantly increased the migration of SUNE1 cells as previously shown, treatment with CYM-5478 did not affect their migratory ability (Figure 5.6). These results were contrasted with the previous observations that HONE1 and SUNE1 cells showed decreased migration following JTE-013 treatment (Figure 5.4), suggesting that S1PR2 might not be the primary receptor involved in S1P-mediated migration of NPC cells.

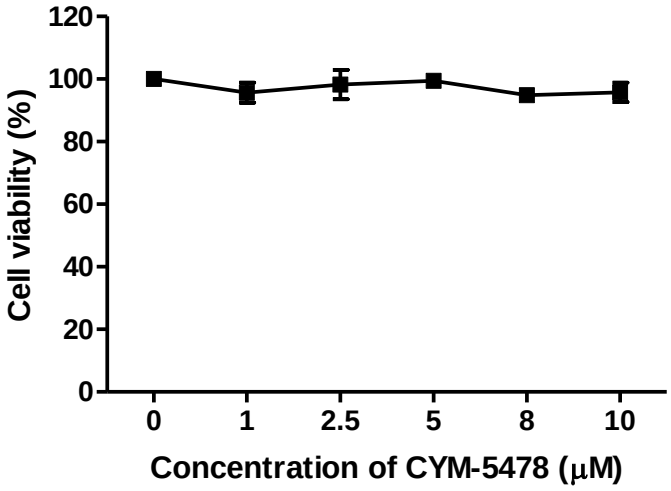


Figure 5.5: NPC cell viability following treatment with CYM-5478 (a S1PR2 agonist)

Treatment of SUNE1 cells with CYM-5478 did not affect cell viability. The data presented are representative of two independent experiments.

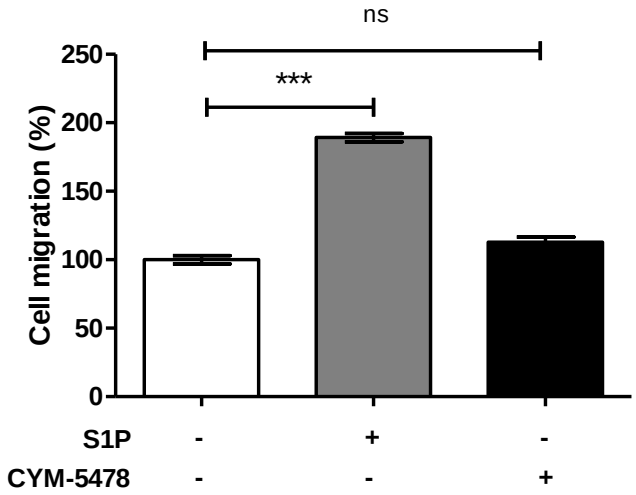


Figure 5.6: Activation of S1PR2 did not increase the migration of SUNE1 cells

The migration of SUNE1 cells was determined using Transwell assays in the absence or presence of S1P (5μM) or CYM-5478 (10μM). While S1P markedly enhanced the migration of SUNE1 cells, no effect was observed following treatment with CYM-5478. The data presented are representative of three independent experiments. *** denotes $p<0.0001$, ns denotes not significant.

5.3.3 VPC23019

To investigate the contribution of S1PR3 to S1P-induced NPC cell migration, NPC cells were treated with a S1PR1/S1PR3 antagonist, VPC23019. VPC23019 acts as a competitive inhibitor of S1P binding to S1PR1 and/or S1PR3 (Davis *et al.*, 2005). The cytotoxicity of VPC23019 in HONE1 and SUNE1 cells was determined by MTT assays. Both cell lines were treated with VPC23019 (1 – 10μM) for 24 hours, and the results showed that concentrations up to 8μM did not affect the viability of these cells (Figure 5.7). 5μM VPC23019 was selected for subsequent migration assays as this concentration has been shown to effectively block the migration of cancer cells such as ovarian cancer, thyroid cancer and hepatocellular carcinoma (Balthasar *et al.*, 2006; Bao *et al.*, 2012; Park *et al.*, 2007).

As shown in Figure 5.8, treatment with VPC23019 alone did not affect the migration of HONE1 and SUNE1 cells in the absence of S1P. However, in the presence of S1P, VPC23019 treatment significantly reduced the migration of HONE1 and SUNE1 cells by 63% and 52%, respectively ($p<0.001$). These results suggested that S1PR1 and/or S1PR3 might involve in the S1P-induced migration of NPC cells.

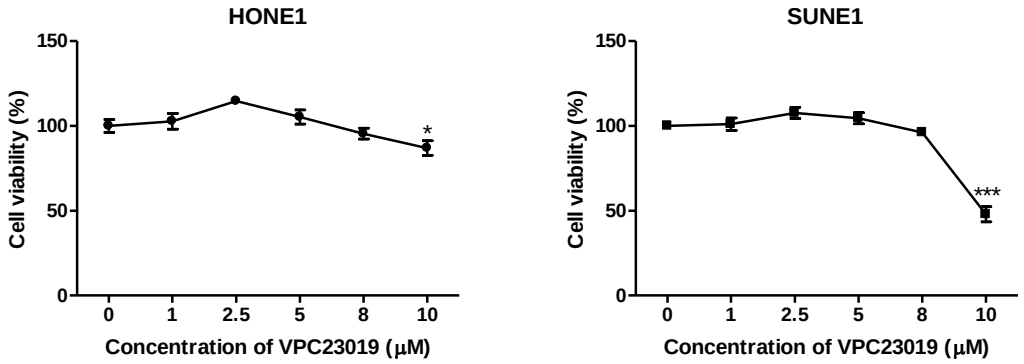


Figure 5.7: NPC cell viability following treatment with VPC23019 (a S1PR1/S1PR3 antagonist)

MTT assays showed that treatment with up to 8μM VPC23019 did not affect the viability of HONE1 and SUNE1 cells. The data presented are representative of two independent experiments. * denotes $p<0.05$, *** denotes $p<0.0001$.

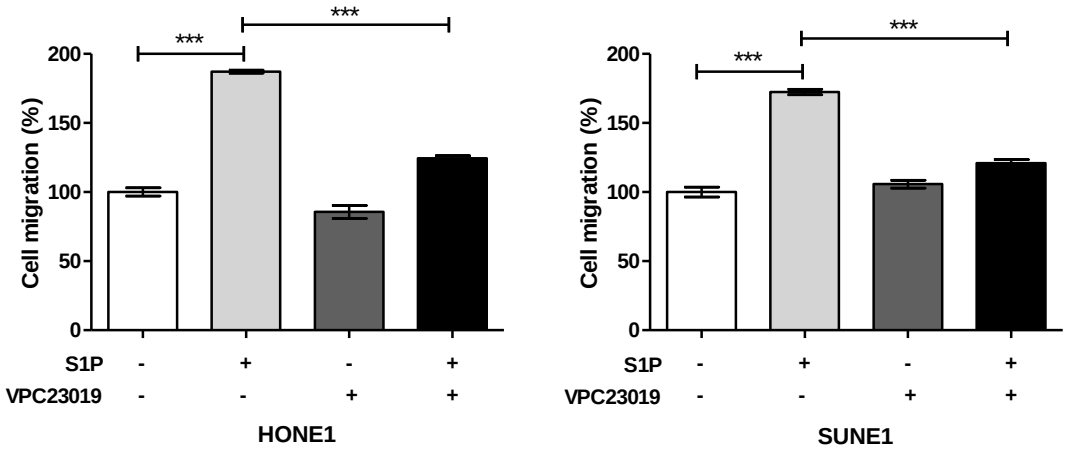


Figure 5.8: Inhibition of S1PR1 and/or S1PR3 suppressed NPC cell migration

The migration of HONE1 and SUNE1 cells was examined using Transwell assays in the absence or presence of S1P (5μM) and/or VPC23019 (5μM). Addition of S1P increased the migration of HONE1 and SUNE1 cells and these effects were significantly suppressed following treatment with VPC23019. The data presented are representative of two independent experiments. *** denotes $p<0.0001$.

5.4 Contribution of S1PR3 to S1P-induced migration

To further explore the contribution of S1PR3 to the S1P-induced migration of NPC cells, knockdown of S1PR3 was performed in SUNE1 cells. SUNE1 cells were selected for these experiments based on their high expression of *S1PR3* (Figure 5.2). To determine the optimum conditions for siRNA transfection, SUNE1 cells were transfected with a pool of four siRNAs against *S1PR3* (siS1PR3) or non-targeting siRNA (NT) at two concentrations (25nM or 50nM) for 48 hours and 72 hours. Compared to the NT, transfection of SUNE1 cells with 25nM or 50nM siS1PR3 significantly reduced the levels of *S1PR3* by approximately 90% at both time points ($p<0.001$; Figure 5.9). 25nM siRNA with a time point of 48 hours was chosen as the condition for subsequent experiments to minimise any possible off-target effect.

Transwell migration assays were then performed to examine the consequence of S1PR3 knockdown on SUNE1 cell migration. In the absence of S1P, the migration of SUNE1/siS1PR3 cells was not statistically different from SUNE1/NT cells (Figure 5.10). In the presence of S1P, knockdown of S1PR3 significantly reduced the migration of SUNE1 cells by 50% ($p<0.001$). These results were consistent with the previous data using VPC23019 (Figure 5.8) and indicate that S1P-induced NPC cell migration was mediated through S1PR3.

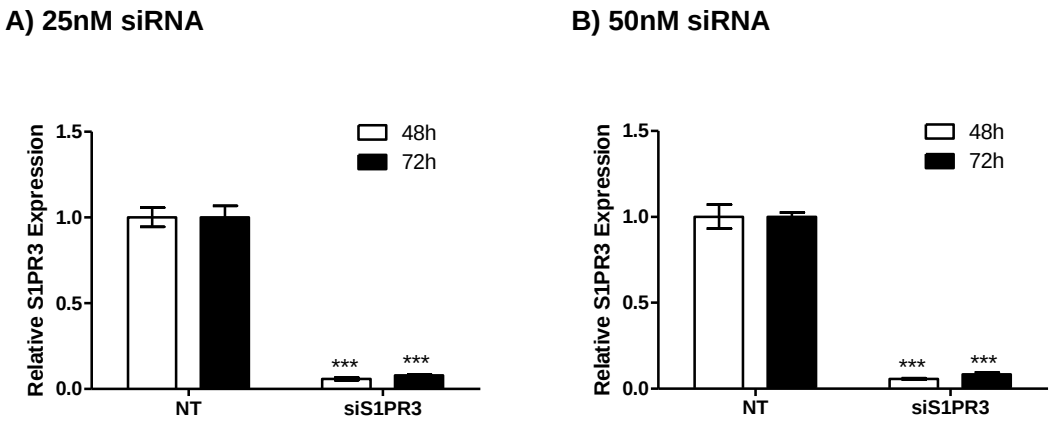


Figure 5.9: Optimisation of the conditions for siRNA knockdown of S1PR3

Compared to SUNE1/NT cells, transfection of (A) 25nM or (B) 50nM of siS1PR3 for 48 hours or 72 hours markedly decreased the expression of *S1PR3*. The data presented are representative of two independent experiments. *** denotes p<0.0001.

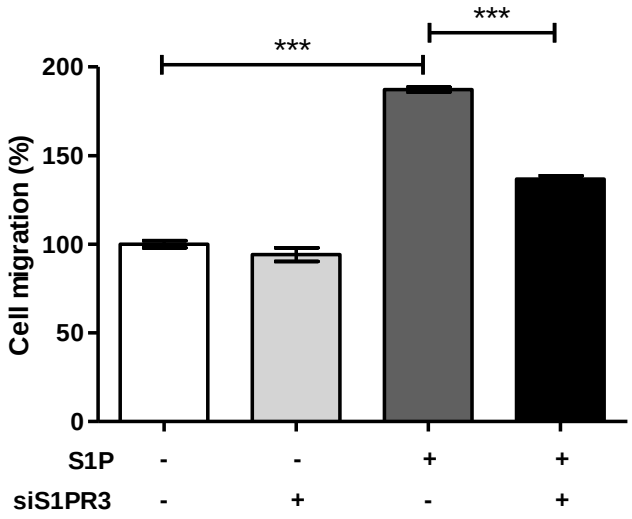


Figure 5.10: Knockdown of S1PR3 inhibited the migration of SUNE1 cells

The migration of SUNE1 cells following transfection of siS1PR3 or NT siRNA was examined using Transwell assays in the absence or presence of S1P (5μM). Addition of S1P significantly promoted the migration of the SUNE1 cells and these effects were suppressed following knockdown of S1PR3. The data presented are representative of three independent experiments. *** denotes p<0.0001.

5.5 The role of S1PR3 and AKT activation in S1P-induced NPC cell migration

Next, I examined whether S1P induced NPC cell migration by activating AKT through S1PR3. Western blot analyses showed that while the levels of the total AKT protein remained unchanged, the phosphorylation of AKT was suppressed following S1PR3 knockdown in SUNE1 cells (Figure 5.11). Furthermore, rescue experiments were performed by co-transfecting SUNE1 cells with siS1PR3 and a constitutively active AKT construct. Western blot analyses confirmed the increased levels of phospho-AKT following the expression of a constitutively active AKT in SUNE1/siS1PR3 cells (Figure 5.12). Transwell migration experiments showed that expression of the constitutively active AKT significantly rescued the suppressive effect of S1PR3 knockdown on the migration of SUNE1 cells ($p<0.001$; Figure 5.13). These results suggested that S1P induces the migration of NPC cells through the activation of AKT via S1PR3.

It has been shown that SPHK1 and S1PR3 form an amplification loop to promote the migration of breast cancer cells (Long *et al.*, 2010a). To examine whether SPHK1 can regulate *S1PR3* expression in NPC cells, Q-PCR analyses were used to determine the expression of *S1PR3* in HONE1 cells following knockdown of SPHK1. However, no change in the *S1PR3* levels was observed in these cells ($p=0.468$; Figure 5.14).

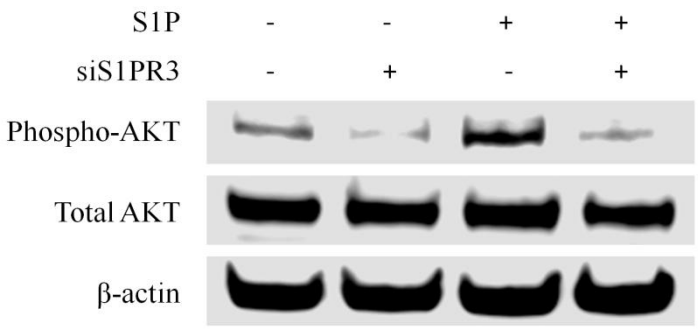


Figure 5.11: Knockdown of S1PR3 in SUNE1 cells inhibited the activation of AKT

Knockdown of S1PR3 did not affect the levels of total AKT protein but markedly suppressed the activation of AKT in SUNE1 cells. Representative western blot images of two independent experiments are presented.

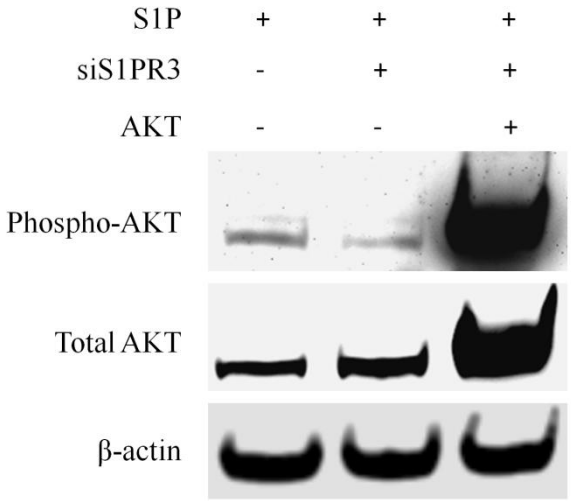


Figure 5.12: Expression of the AKT protein following transfection of SUNE1 cells with a constitutively active AKT

Western blot analyses confirmed the increased levels of both total and phospho-AKT proteins in SUNE1 cells co-transfected with siS1PR3 and a constitutively active AKT construct. Representative western blot images of two independent experiments are presented.

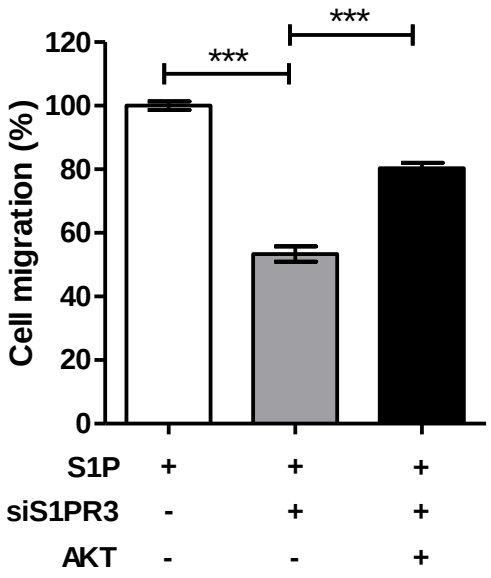


Figure 5.13: Expression of a constitutively active AKT rescued the suppressive effect of S1PR3 knockdown on the migration of SUNE1 cells

The migration of SUNE1 cells following S1PR3 knockdown and expression of a constitutively active AKT was examined using Transwell assays in the presence of S1P (5μM). While knockdown of S1PR3 significantly decreased the migration of SUNE1 cells, these effects were partially reversed following expression of constitutively active AKT. The data presented are representative of three independent experiments. *** denotes p<0.0001.

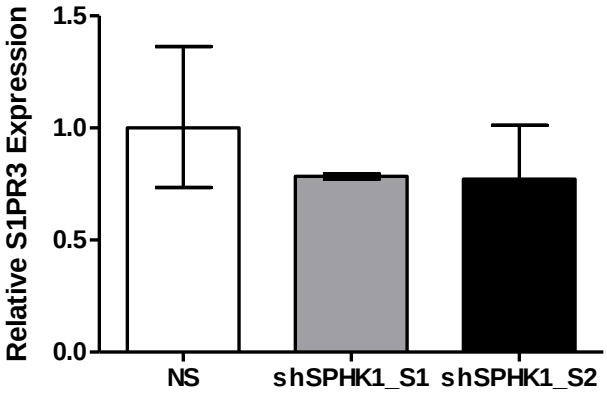


Figure 5.14: Knockdown of SPHK1 did not affect the expression of *S1PR3*

Compared to NS, the expression of *S1PR3* was not significantly different following knockdown of SPHK1 in HONE1 cells (p=0.468). The data presented are representative of two independent experiments .

5.6 Summary

The re-analyses of two published microarray datasets (GSE12452 and GSE34573) revealed significant and consistent up-regulation of *S1PR3* in micro-dissected NPC cells compared to normal epithelium. Upregulation of *S1PR2* and *S1PR5* in NPC cells was only evident in the GSE12452 dataset. Q-PCR profiling of the five S1P receptors in a panel of eight NPC cell lines showed that *S1PR2* and *S1PR5* mRNAs were readily detected in all cell lines whilst the expression of *S1PR3* was variable. Since limited reagents are available for the studies of S1PR5, only the roles of S1PR2 and S1PR3 were studied in subsequent experiments.

In order to examine the contribution of S1PR2 to S1P-mediated migration of NPC cells, a S1PR2 antagonist (JTE-013) and an agonist (CYM-5478), were used. Following treatment with JTE-013, the migration of HONE1 and SUNE1 cells was significantly inhibited in the presence of S1P. However, addition of CYM-5478 did not affect the migration of SUNE1 cells. These contradictory data implied that S1PR2 might not be the primary receptor involved in the S1P-mediated NPC cell migration.

To determine the involvement of S1PR3 in S1P-induced NPC cell migration, the S1PR1/S1PR3 antagonist, VPC23019, and siRNA knockdown of S1PR3 were used. In the presence of S1P, the migration of HONE1 and SUNE1 cells was significantly reduced following addition of VPC23019. These results were supported by S1PR3 knockdown in SUNE1 cells, in which reduction of S1PR3 expression markedly suppressed the S1P-induced migration of SUNE1 cells. These findings suggested that S1P enhanced NPC cell migration through S1PR3.

Next, the association of S1P/S1PR3 and AKT activation in NPC cell migration was investigated. Knockdown of S1PR3 in SUNE1 cells was accompanied by a reduction in the levels of phospho-AKT and the expression of a constitutively active AKT restored

the suppressive effect of S1PR3 knockdown on the migration of these cells. Collectively, these data demonstrate that S1P induced the migration of NPC cells, at least in part, via the activation of AKT through S1PR3.

CHAPTER 6:

CONTRIBUTION OF EBV INFECTION TO THE EXPRESSION OF SPHK1 AND S1PR3

6.1 Introduction

Non-keratinising NPC is consistently associated with EBV infection (Niedobitek, 2000). In endemic regions, EBV genomes are detected in almost all NPC cases regardless of the histopathological type (Pathmanathan *et al.*, 1995). EBV establishes type II latency in NPC in which EBV gene expression is restricted to EBNA1, LMP1, LMP2, EBERs, BARTs and BARF1 (Young *et al.*, 2014).

It has been shown that immortalised nasopharyngeal epithelial cells displaying pre-malignant genetic changes (e.g. p16 deletion or overexpression of cyclin D1) are susceptible to EBV infection (Tsang *et al.*, 2012). Once infected, EBV genes provide growth and survival benefits by inducing additional alterations leading to the development of NPC (Tsao *et al.*, 2015). In particular, EBV genes such as LMP1 and LMP2A exhibit oncogenic properties and activate a number of signalling pathways in NPC, including NF-κB, PI3K/AKT and MAPK/ERK (Young *et al.*, 2014). Despite the fact that EBV infection is closely linked to NPC, the exact contribution of EBV to the pathogenesis of NPC remains enigmatic.

From the Q-PCR analyses, the only EBV-positive NPC cell line, C666-1, expressed the highest levels of *SPHK1* (Figure 4.5) and *S1PR3* (Figure 6.1) compared to a panel of EBV-negative NPC cell lines and immortalised nasopharyngeal cells, implying that EBV infection might regulate the expression of these genes. The aims of this study were: 1) to establish EBV-infected NPC cell lines and, 2) to use these cell lines to determine the contribution of EBV infection to the expression of SPHK1 and S1PR3 in NPC cells.

6.2 Establishment of EBV-infected NPC cell lines

Two NPC cell lines, CNE1 and TW04, were selected to be stably infected with a recombinant EBV (Akata strain) based on their low levels of *SPHK1* (Figure 4.5) and *S1PR3* (Figure 6.1). A Burkitt's lymphoma-derived cell line that carries the recombinant EBV, Akata, was used and EBV virions were produced by inducing the lytic cycle using an anti-human IgG antibody (Shimizu *et al.*, 1996). EBV-infected CNE1 (CNE1/EBV) and TW04 (TW04/EBV) cells were established by co-culturing with the induced Akata cells, followed by selection in G418 for 7 days.

To determine whether CNE1/EBV and TW04/EBV cells exhibit EBV type II latency, Q-PCR and immunofluorescence analyses were performed to examine the mRNA and protein levels of the EBV latent genes (EBNA1, LMP1 and LMP2A), respectively. The results showed that EBNA1 and LMP2A mRNA (Figure 6.2) and protein (Figure 6.3) were expressed in both CNE1/EBV and TW04/EBV cells, but LMP1 was not detectable in either cell line.

To compare the EBV gene expression pattern in CNE1/EBV and TW04/EBV cells with those in other established EBV(Akata)-infected NPC cell lines, Q-PCR analyses were performed in EBV-infected CNE2, HK1, HONE1, SUNE1 and TW01 cells (gifts from Dr Christopher Dawson, University of Birmingham and Prof George Tsao, University of Hong Kong). Similar expression patterns were observed in these cells, in which the levels of *EBNA1* and *LMP2A* mRNA were readily detected whereas *LMP1* was not expressed (Figure 6.4). These results confirmed that LMP1 expression is rarely detected in NPC cells stably infected with the Akata EBV strain.

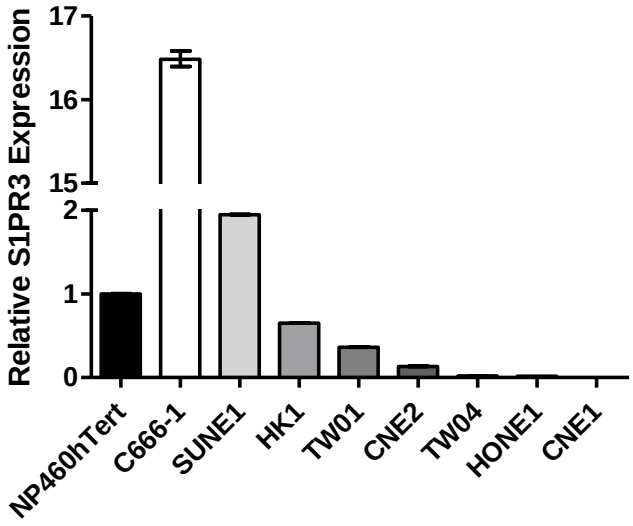


Figure 6.1: Expression of *S1PR3* in NPC cell lines

The expression of *S1PR3* in NPC cell lines and an immortalised nasopharyngeal cell line, NP460hTert was determined by QPCR. The highest levels of *S1PR3* were detected in C666-1, the only EBV-positive NPC cell line. The data presented are representative of two independent experiments.

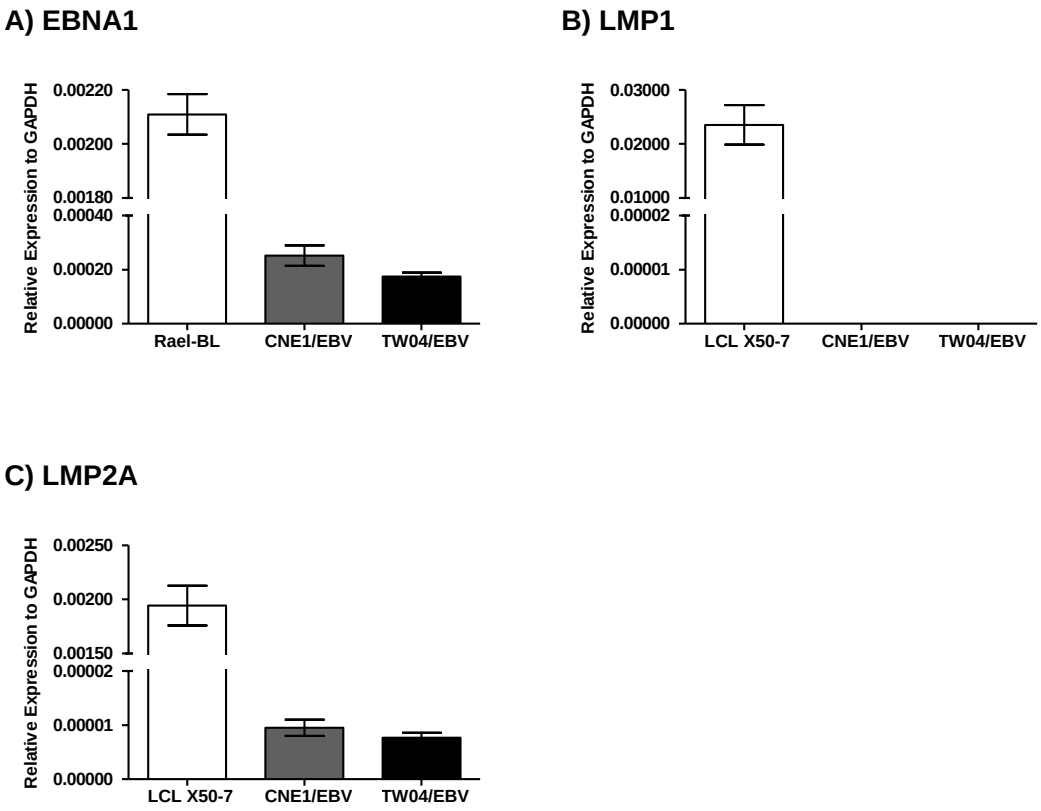


Figure 6.2: Expression of EBV latent genes in EBV-infected CNE1 and TW04

Expression of (A) *EBNA1* and (C) *LMP2A*, but not (B) *LMP1*, was detected in CNE1 and TW04 cells stably infected with a recombinant EBV (Akata strain) by QPCR. Rael-BL served as a positive control for EBNA1 whereas LCL X50-7 served as a positive control for LMP1 and LMP2A. The data presented are representative of two independent experiments.

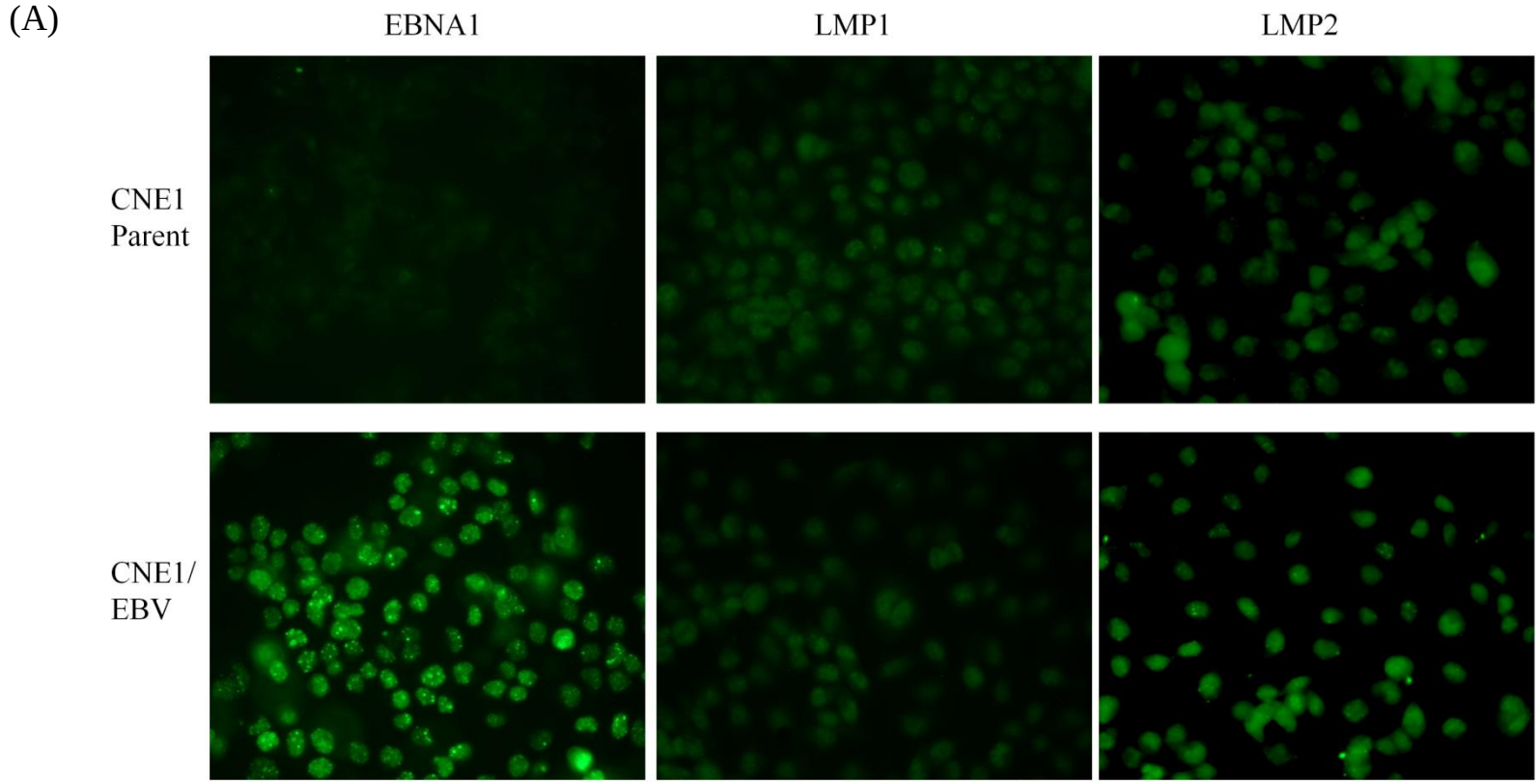


Figure 6.3: Expression of EBV latent proteins in EBV-infected CNE1 and TW04 cells

Immunofluorescence analyses showed that (A) CNE1/EBV and (B) TW04/EBV cells expressed only EBNA1 and LMP2 proteins, but not LMP1 protein.

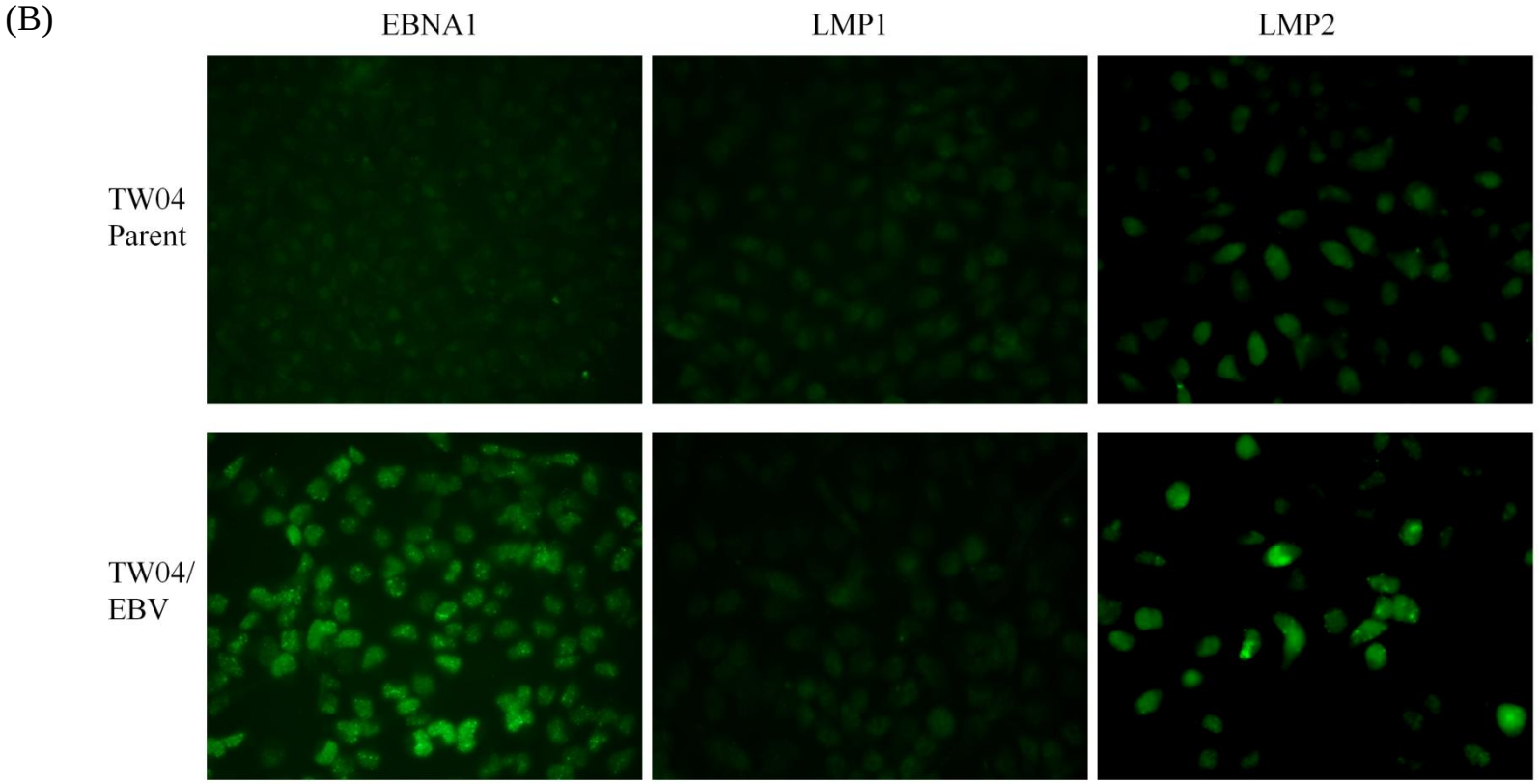


Figure 6.3, continued

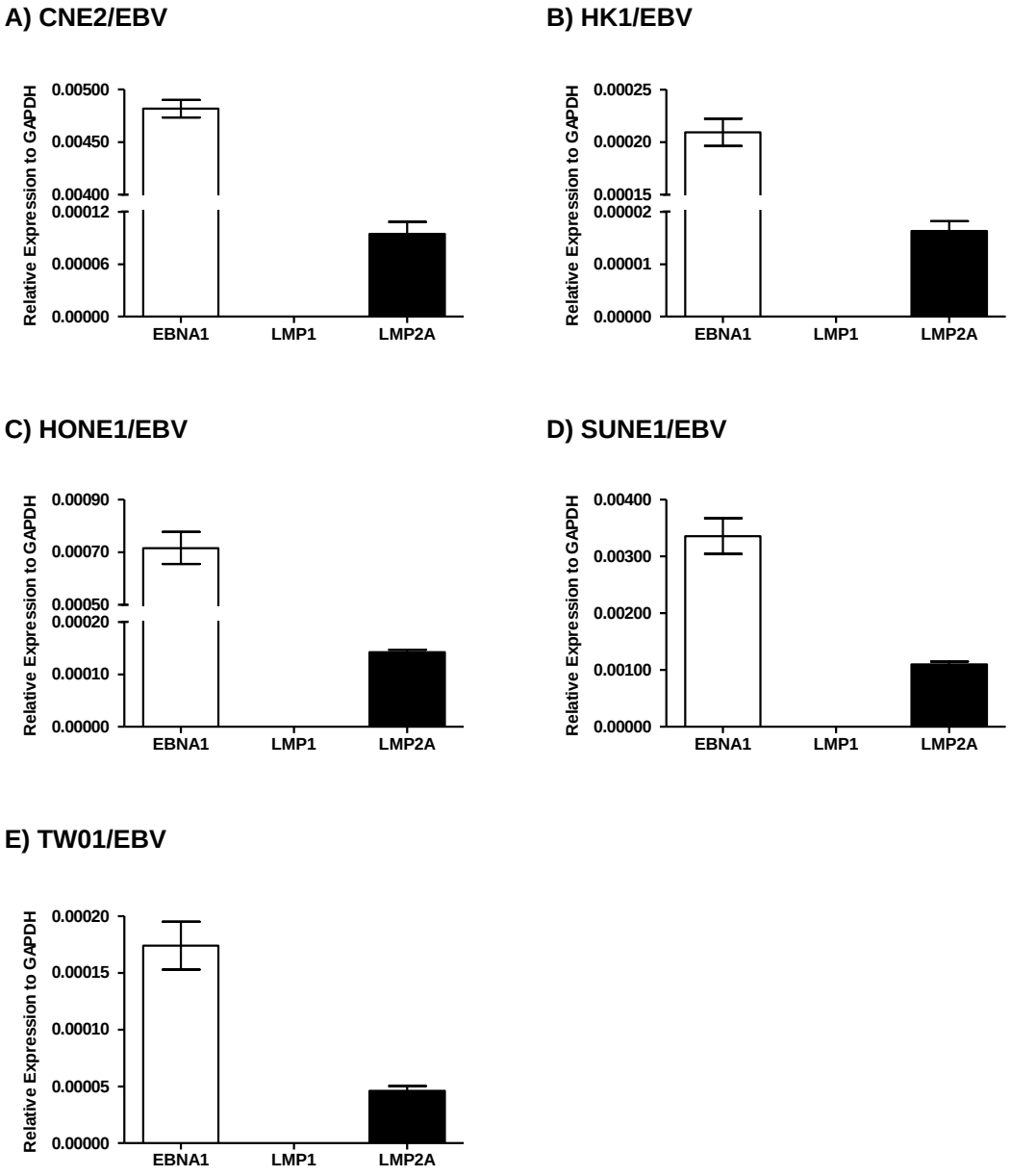


Figure 6.4: Expression of EBV latent genes in EBV-infected NPC cells

Similar to CNE1/EBV and TW04/EBV cells, only *EBNA1* and *LMP2A*, but not LMP1, were detected in EBV-infected (A) CNE2, (B) HK1, (C) HONE1, (D) SUNE1 and (E) TW01 cells using QPCR. The data presented are representative of two independent experiments.

6.2.1 Phenotypic characteristics of CNE1/EBV and TW04/EBV cells

In order to determine whether CNE1/EBV and TW04/EBV cells possess similar phenotypic properties as other EBV-infected NPC cells, the proliferation, migratory and invasive potential of these cells were assessed. Similar to the previously established HK1/EBV cells (Lo *et al.*, 2006), the growth of CNE1/EBV and TW04/EBV cells was significantly slower than their respective parental controls (Figure 6.5).

It is known that EBV infection promotes the migration and invasion of NPC cells (Lui *et al.*, 2009; Teramoto *et al.*, 2000; Wu *et al.*, 2003). Consistent with this concept, the migration (Figure 6.6) and invasion (Figure 6.7) of CNE1/EBV and TW04/EBV cells were significantly increased compared to their respective parental controls ($p<0.001$). These results suggested that CNE1/EBV and TW04/EBV cells behaved similarly to other established EBV-infected cell lines and can be used as *in vitro* models to study the roles of EBV infection in NPC cells.

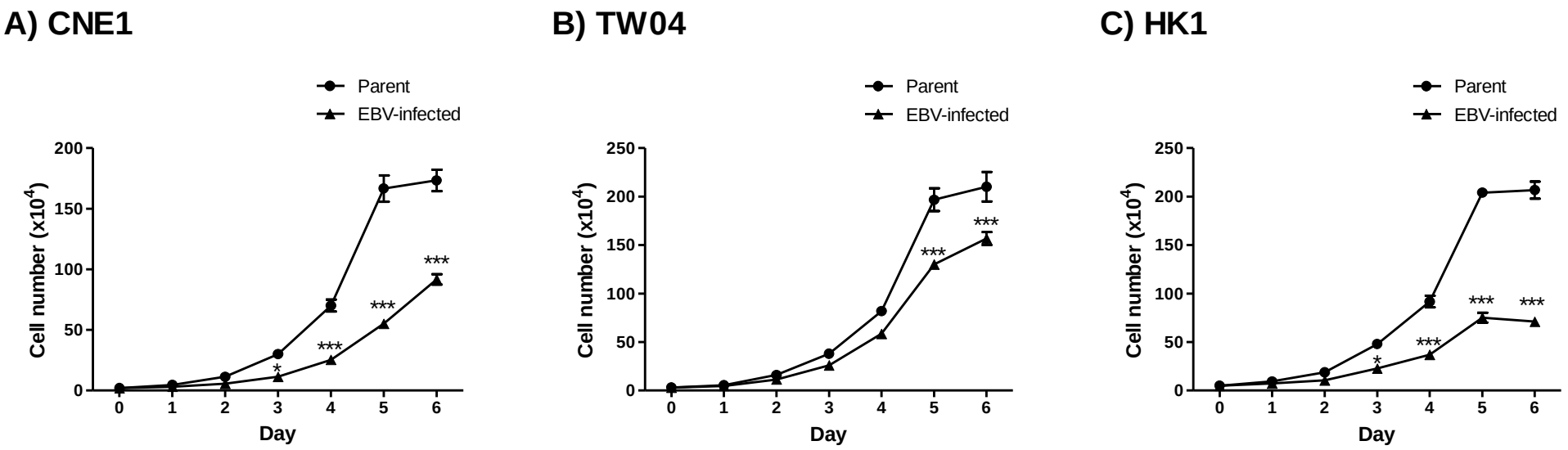


Figure 6.5: EBV infection reduced NPC cell proliferation

Compared to respective parental controls, (A) CNE1, (B) TW04 and (C) HK1 cells grew slower following EBV infection. The data presented are representative of two independent experiments. * denotes $p<0.05$, *** denotes $p<0.001$.

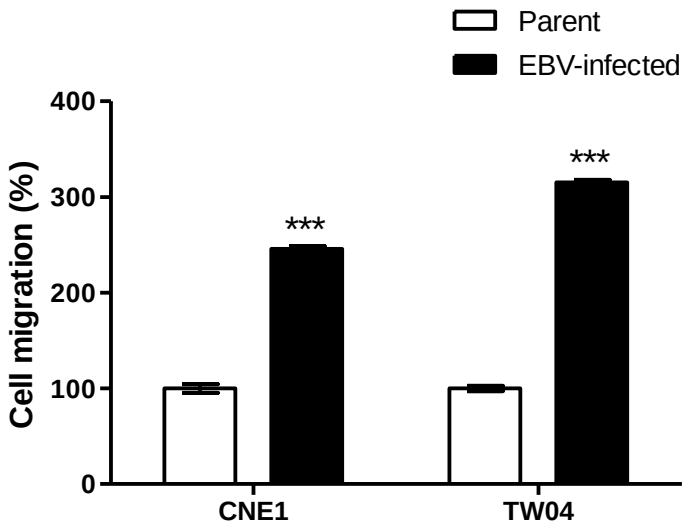


Figure 6.6: EBV infection promoted NPC cell migration

The migration of CNE1/EBV and TW04/EBV cells were examined using Transwell assays in the presence of 10% FBS in the lower chambers. The migration of CNE1/EBV and TW04/EBV cells was markedly higher compared to their respective parental controls. The data presented are representative of two independent experiments. *** denotes $p<0.0001$.

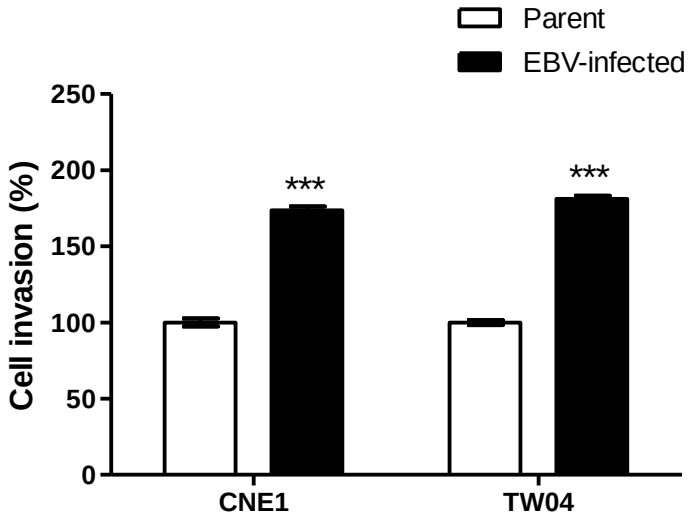


Figure 6.7: EBV infection increased NPC cell invasion

The invasion of CNE1/EBV and TW04/EBV cells was determined using matrigel-coated Transwell assays in the presence of 10% FBS in the lower chambers. CNE1/EBV and TW04/EBV cells showed increased invasive potential compared to their respective parental controls. The data presented are representative of two independent experiments. *** denotes $p<0.0001$.

6.3 Contribution of EBV infection to the expression of SPHK1 and S1PR3

Seven EBV-infected NPC cell lines (CNE1, CNE2, HK1, HONE1, SUNE1, TW01 and TW04) and an EBV-infected immortalised nasopharyngeal cell line (NP460hTert) were used to determine whether EBV can regulate the expression of SPHK1 and S1PR3.

6.3.1 SPHK1

Q-PCR analyses showed that compared to the respective parental cells, the expression of *SPHK1* mRNA was significantly increased in six of the eight EBV-infected cell lines (HK1, CNE1, SUNE1, HONE1, CNE2 and TW01) whereas no change was detected in NP460hTert/EBV and TW04/EBV cells (Figure 6.8). Western blot analyses showed that the expression of both the total and phosphorylated SPHK1 proteins was elevated in HK1/EBV, CNE1/EBV, SUNE1/EBV, HONE1/EBV and CNE2/EBV cells, but unchanged in TW01/EBV cells (Figure 6.9).

6.3.2 S1PR3

In contrast to SPHK1 expression, following EBV infection, the expression of *S1PR3* was only increased in three (HK1, TW04 and NP460hTert) out of the eight cell lines examined and decreased in the other three cell lines (CNE2, SUNE1 and HONE1). The levels of *S1PR3* were unchanged in TW01/EBV and CNE1/EBV cells (Figure 6.10).

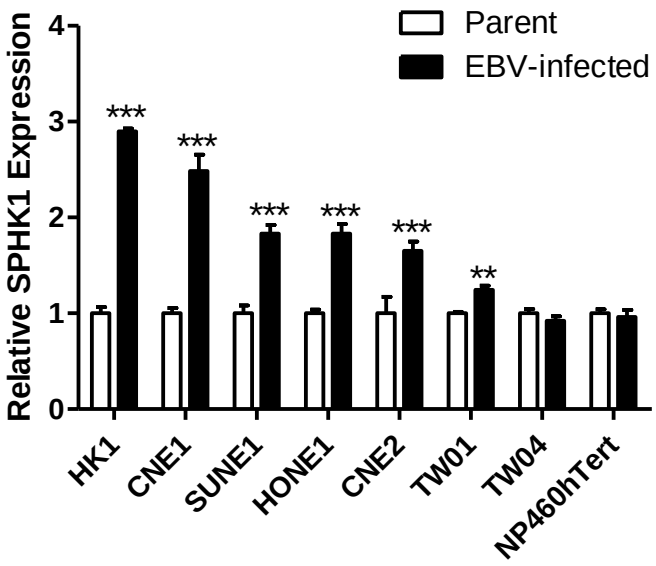


Figure 6.8: EBV infection increased the mRNA expression of *SPHK1* in NPC cells

Q-PCR analyses showed that six out of the eight EBV-infected cell lines expressed higher levels of *SPHK1* than their respective parental controls. The data presented are representative of two independent experiments. ** denotes $p<0.01$; *** denotes $p<0.001$.

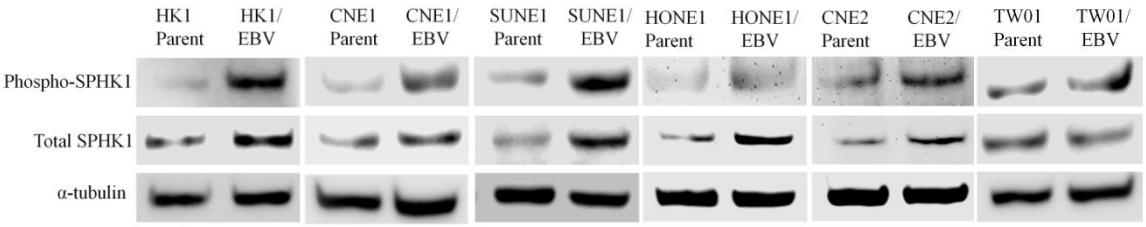


Figure 6.9: EBV infection upregulated both the total and phosphorylated SPHK1 proteins in NPC cells

Among the six NPC cell lines that showed elevated SPHK1 mRNA levels following EBV infection, five of them (HK1, CNE1, SUNE1, HONE1 and CNE2) also demonstrated an increase in the expression of both total and phosphorylated SPHK1 proteins. Representative western blot images of two independent experiments are presented.

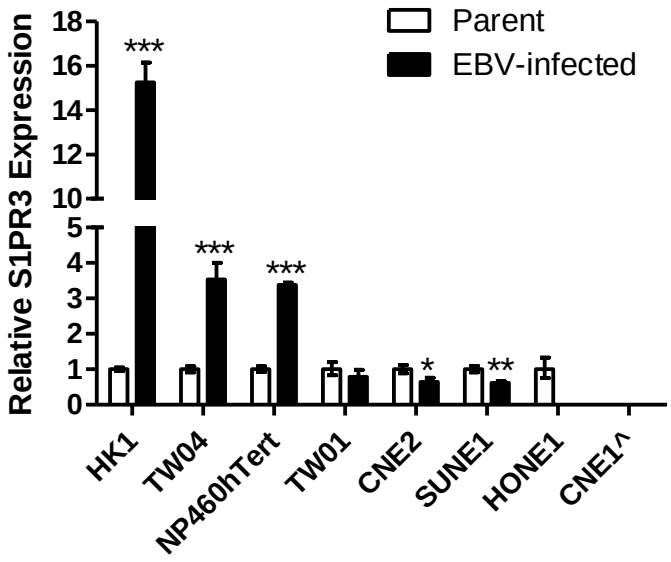


Figure 6.10: Expression of *S1PR3* in nasopharyngeal cell lines following EBV infection

Compared to the respective parental controls, Q-PCR analyses showed that following EBV infection, three out of eight cell lines (HK1, TW04 and NP460hTert) expressed higher levels of *S1PR3* whilst CNE2, SUNE1 and HONE1 showed lower expression of *S1PR3*. The levels of *S1PR3* were unchanged in TW01/EBV and CNE1/EBV. ^ Expression of *S1PR3* was not detected in either CNE1 or CNE1/EBV cells. The data presented are representative of two independent experiments. * denotes $p<0.05$; ** denotes $p<0.01$, *** denotes $p<0.001$.

6.3.3 Correlation between SPHK1 and S1PR3 expression

Among the eight cell lines, only HK1 cells consistently showed the upregulation of both *SPHK1* and *S1PR3* following EBV infection (Table 6.1). Spearman's correlation analysis was performed to determine the association of *SPHK1* and *S1PR3* expression in the other seven EBV-infected cell lines. A significant negative correlation was found in the expression of *SPHK1* and *S1PR3* in these cells (Spearman's rho = -0.9727, $p<0.01$) (Figure 6.11), indicating that EBV-mediated upregulation of *SPHK1* and *S1PR3* expression could be mutually exclusive in nasopharyngeal epithelial cells.

Table 6.1: Significant changes in the levels of *SPHK1* and *S1PR3* following EBV infection

Cell lines	Changes in *SPHK1* levels following EBV infection	Changes in *S1PR3* levels following EBV infection
HK1	Upregulated	Upregulated
CNE2	Upregulated	Downregulated
HONE1	Upregulated	Downregulated
SUNE1	Upregulated	Downregulated
CNE1	Upregulated	No change
TW01	Upregulated	No change
TW04	No change	Upregulated
NP460hTert	No change	Upregulated

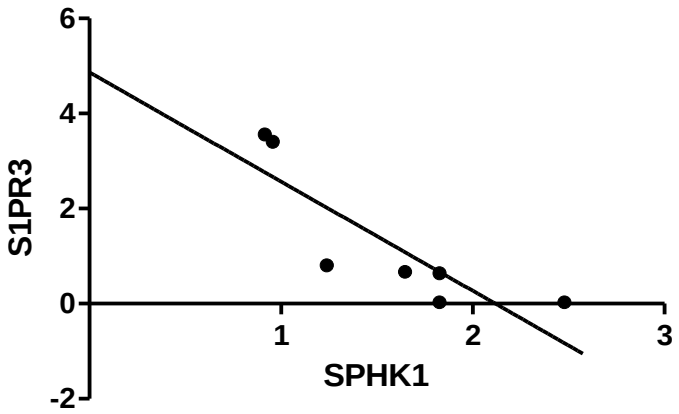


Figure 6.11: Correlation of the *SPHK1* and *S1PR3* expression

The expression of *SPHK1* is negatively correlated with the expression of *S1PR3* in EBV-infected cells.

6.4 Expression of SPHK1 and S1PR3 following transfection of individual EBV latent genes

To determine which EBV latent gene was responsible for the upregulation of *SPHK1*, HONE1 cells stably transfected with EBNA1, LMP1 or LMP2A gene (Port *et al.*, 2013) and HK1 cells transiently transfected with these latent genes were used. Transiently transfected HK1 cells were also used to examine the regulation of S1PR3 expression.

6.4.1 SPHK1

Q-PCR analyses showed that compared to the vector controls, *SPHK1* mRNA levels were significantly elevated in HONE1 cells stably transfected with EBNA1, LMP1 or LMP2A (Figure 6.12). In agreement with the changes in mRNA levels, the expression of both total and phosphorylated SPHK1 proteins were also increased in these cells (Figure 6.13). These results suggested that EBNA1, LMP1 and LMP2A could upregulate the expression of SPHK1 in NPC cells.

Since LMP1 and LMP2A are known to promote the migration of NPC cells (Tsao *et al.*, 2015), the contribution of these two EBV latent genes to the expression of SPHK1 was further determined in HK1 cells transiently transfected with LMP1 or LMP2A. Q-PCR analyses confirmed the expression of LMP1 and LMP2A in the transfected HK1 cells (Figure 6.14). The levels of *SPHK1* remained unchanged in these cells (Figure 6.15), showing that LMP1 and LMP2A did not upregulate the expression of SPHK1 in HK1 cells.

6.4.2 S1PR3

Although EBV infection of HK1 cells enhanced the expression of S1PR3 (Figure 6.11), transient transfection of HK1 with LMP1 or LMP2A did not alter the expression levels of S1PR3 (Figure 6.16).

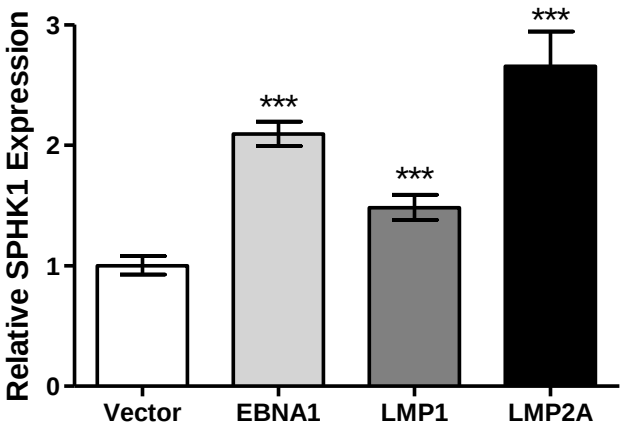


Figure 6.12: EBV latent genes upregulated the expression of *SPHK1* in NPC cells

Compared to the vector control, increased expression of *SPHK1* was detected in HONE1 cells stably transfected with EBV latent genes (EBNA1, LMP1 or LMP2A). The data presented are representative of three independent experiments. *** denotes $p<0.001$.

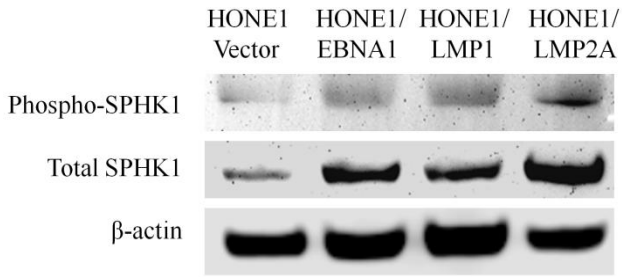


Figure 6.13: EBV latent genes increased both the total and phosphorylated SPHK1 proteins in NPC cells

HONE1 cells stably transfected with EBNA1, LMP1 or LMP2A genes exhibited upregulated levels of phospho-SPHK1 and total SPHK1 proteins compared to the vector control. Representative western blot images of two independent experiments are shown.

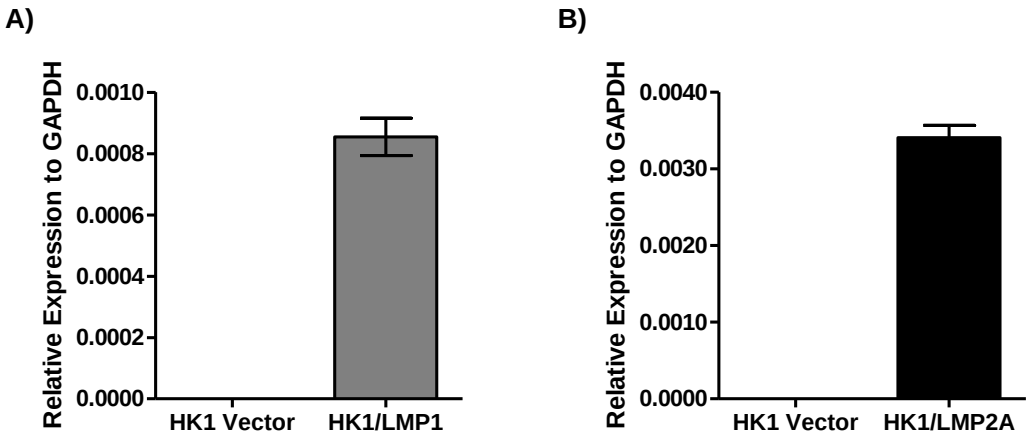


Figure 6.14: Confirmation of *LMP1* and *LMP2A* expression in transfected HK1 cells

Q-PCR analyses confirmed the expression of (A) *LMP1* and (B) *LMP2A* in HK1 cells transiently transfected with LMP1 and LMP2A for 72 hours. The data presented are representative of two independent experiments.

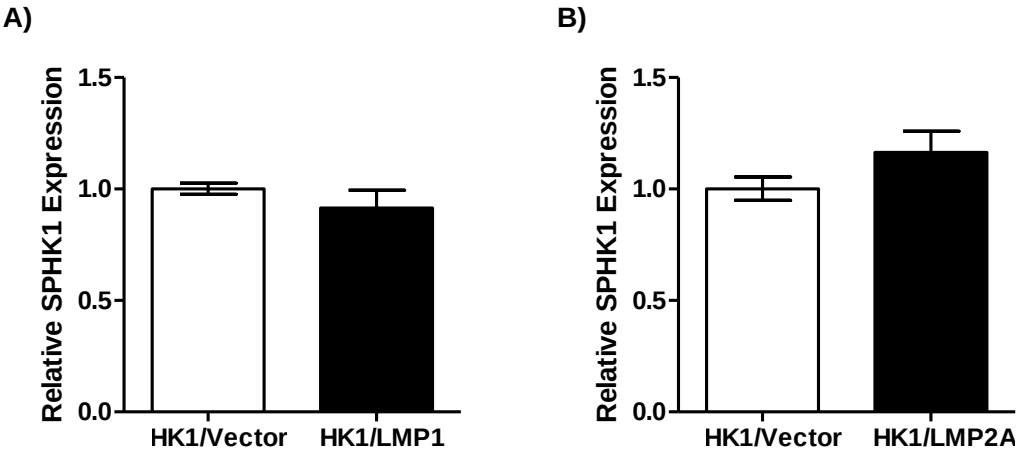


Figure 6.15: Expression of *SPHK1* in LMP1- and LMP2A-transfected HK1 cells

Compared to the vector control, the levels of *SPHK1* were unchanged in transiently (A) LMP1- and (B) LMP2A-transfected HK1 cells. The data presented are representative of two independent experiments.

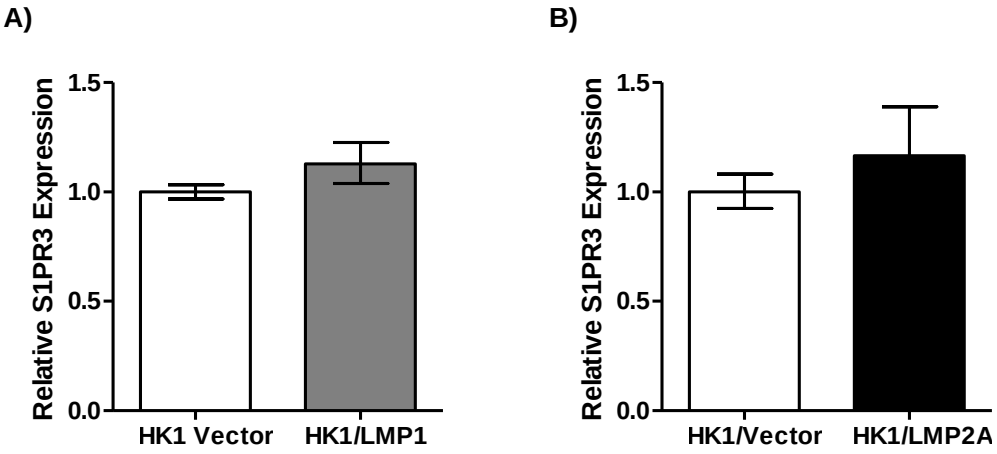


Figure 6.16: Expression of *S1PR3* in LMP1- and LMP2A-expressing HK1 cells

Compared to the vector control, the expression of *S1PR3* was not significantly altered in HK1 cells following transient transfection of (A) LMP1 and (B) LMP2A. The data presented are representative of two independent experiments.

6.5 Summary

Among a panel of NPC cell lines, C666-1, the only EBV-positive NPC cell line, showed the highest levels of *SPHK1* and *S1PR3*, implying that EBV infection could upregulate the expression of *SPHK1* and *S1PR3*. To generate *in vitro* models to study the role of EBV infection in NPC, CNE1 and TW04 cells were stably infected with a recombinant EBV (Akata strain) using the established cell-to-cell contact protocol. Similar to other previously established EBV-infected cells, EBNA1 and LMP2, but not LMP1, were expressed in CNE1/EBV and TW04/EBV cells. In addition, CNE1/EBV and TW04/EBV cells grew slower but had increased migratory and invasive properties compared to their respective parental controls. These phenotypic characteristics were also found in the other established EBV-infected NPC cells, demonstrating that CNE1/EBV and TW04/EBV cells could be used as additional *in vitro* models for studies of EBV infection in NPC cells.

Among the eight EBV-infected nasopharyngeal epithelial cells (two newly established and six obtained from collaborators), EBV infection upregulated both the mRNA and protein expression of SPHK1 in five of the cell lines (HK1, CNE1, SUNE1, HONE1 and CNE2) whereas the expression of *S1PR3* was only increased in three of these cell lines (HK1, TW04 and NP460hTert) and decreased in another three cell lines (CNE2, SUNE1 and HONE1). Spearman's correlation analyses revealed a negative correlation of *SPHK1* and *S1PR3* expression (Spearman's rho = -0.9727) in these cells, implying that the upregulation of *SPHK1* or *S1PR3* modulated by EBV might be mutually exclusive in nasopharyngeal epithelial cells.

To determine which EBV latent gene was responsible for the expression of SPHK1, HONE1 cells stably transfected with EBV latent genes (EBNA1, LMP1 or LMP2A) were used. All these EBV latent genes were found to upregulate the mRNA and protein

levels of SPHK1. The contribution of LMP1 and LMP2A to the expression of *SPHK1* and *S1PR3* was also determined in HK1 cells transiently transfected with these two EBV genes. However, the levels of *SPHK1* and *S1PR3* were unchanged in LMP1- and LMP2A-expressing HK1 cells compared to the controls, suggesting that the effects of EBV latent genes are context dependent.

CHAPTER 7: DISCUSSION

7.1 Introduction

It is evident that S1P signalling plays an important role in promoting tumorigenesis in a wide range of cancer types by increasing cancer cell proliferation, survival, migration, invasion and angiogenesis (Takabe *et al.*, 2014). Targeting SPHK1, S1P itself and/or S1PRs is now a promising new cancer therapeutic strategy (Kunkel *et al.*, 2013). Although high expression of SPHK1 was shown to be associated with poorer survival in NPC patients (Li *et al.*, 2015b), the biological consequence of aberrant activation of S1P signalling in the pathogenesis of NPC has not been studied. Therefore, the present study was initiated to investigate how aberrant S1P signalling influences the malignant phenotypes of NPC cells and whether EBV infection contributes to the dysregulation of this pathway. Since elevated expression of SPHK1 has been detected in primary NPCs (Li *et al.*, 2015b), high levels of S1P are likely to be present in NPC tissues. The results of this study showed that exogenous S1P increased the migration of NPC cells, whilst knockdown of SPHK1 suppressed cell migration. Next, the mechanisms of S1P-induced migration were elucidated and S1P was shown to induce NPC cell migration by activating AKT via S1PR3. Lastly, EBV infection was found to be able to upregulate the expression of SPHK1 and S1PR3 in NPC cell lines.

For ease of interpretation, this Discussion chapter has been subdivided such that consideration is given to the data reported in each results chapter. The following sections discuss some of the limitations of this study and present proposals for future work.

7.2 Phenotypic impact of exogenous S1P and knockdown of SPHK1

Over-expression of SPHKs has been shown to transform NIH3T3 cells and promote tumour formation in NOD/SCID mice, pointing a role for SPHKs as an oncogene (Xia

et al., 2000). High expression of SPHK1 is reported in various types of cancers, and correlates with a number of clinicopathological parameters, including advanced stage, metastasis and shorter patient survival (Pyne *et al.*, 2010). SPHK1 was found to be highly expressed in primary NPC tissues (Li *et al.*, 2015b), hence high levels of S1P are likely to be present in NPC cells. In the first part of this study, the biological function of exogenous S1P on NPC cell proliferation, migration and invasion was examined. The effects of inhibiting endogenous SPHK1 were examined by knocking down SPHK1 in NPC cells using a lentiviral shRNA system to achieve a stable and long-term knockdown of SPHK1. SPHK1 produces S1P following activation through phosphorylation by ERK1/2 (Pitson *et al.*, 2003). A reduction in the levels of both total and phosphorylated SPHK1 proteins was demonstrated following SPHK1 knockdown, indicating that the activated form of SPHK1 was decreased and this was likely to result in a reduction in S1P levels.

7.2.1 Effects on cell proliferation

Whilst S1P is generally considered to stimulate the growth of cancer cells through the activation of several signalling pathways including PI3K/AKT and MAPK/ERK (Datta *et al.*, 2014; Nava *et al.*, 2002; Van Brocklyn *et al.*, 2002; Xia *et al.*, 2012), there are also reports to demonstrate an anti-proliferative role for this lipid in cancer (Balthasar *et al.*, 2006; Hong *et al.*, 1999; Ling *et al.*, 2011; Shin *et al.*, 2007; Yamashita *et al.*, 2006). Although there is a paucity of information on how S1P inhibits the growth of cancer cells, the possible mechanisms could be inferred from data in non-malignant cells. For example, S1P inhibited the proliferation of human keratinocytes through rapid phosphorylation of Smad3 and interaction with Smad4 independently of transforming growth factor-beta (TGF-β) secretion (Sauer *et al.*, 2004). S1P can also suppress the growth of hepatic myofibroblasts by rapidly increasing prostaglandin E2 production that in turn elevated the production of a growth inhibitory messenger, cyclic

adenosine monophosphate (cAMP) (Davaille *et al.*, 2000). In the present study, exogenous addition of S1P consistently inhibited the growth of four NPC cell lines (CNE1, HK1, HONE1 and TW04). However, knockdown of SPHK1 also reduced the proliferation of HONE1 cells. There are two possible explanations for these contradictory results. Firstly, addition of S1P might disrupt the sphingolipid rheostat such that excess S1P was converted to pro-apoptotic sphingosine and ceramide (Taha *et al.*, 2006b). Ceramide has been shown to induce cell cycle arrest by dephosphorylating retinoblastoma protein (Dbaibo *et al.*, 1995), inactivating CDK2 (Lee *et al.*, 2000) as well as activating p21 (Phillips *et al.*, 2007) and p27 (Zhu *et al.*, 2003). This possibility could be examined by determining the levels of ceramide, sphingosine and S1P in NPC cells following S1P addition. Secondly, the reduction in S1P levels following SPHK1 knockdown might not be sufficient to overcome the anti-mitogenic effects of endogenous S1P (Igarashi *et al.*, 2003; Maceyka *et al.*, 2005) and this speculation could be tested by measuring the levels of S1P in HONE1 cells following SPHK1 knockdown.

7.2.2 Effects on migration and invasion

Cell migration and invasion are key events in cancer metastasis. Given that NPC is a highly metastatic cancer and distant metastasis is the major cause of death in NPC patients, this study examined the effects of S1P on the migratory and invasive potential of NPC. The results showed that S1P treatment consistently enhanced the migration of three NPC cell lines (HONE1, SUNE1 and TW04) and also the invasion of SUNE1 cells. In line with these data, knockdown of SPHK1 significantly suppressed the migration of HONE1 cells. These results were in agreement with the general notion that aberrant activation of S1P signalling enhances the migration and invasion of cancer cells and is associated with cancer metastasis *in vivo* (Brocklyn, 2010).

7.3 Identification of the S1P receptors that mediate S1P-induced migration in NPC cells

S1P acts as a specific ligand for the five S1P receptors, S1PR1 – S1PR5 (Rivera *et al.*, 2008). A large body of evidence has reported the deregulation of S1PRs in cancer and the effects of S1P are dependent on the expression profile of S1PRs (Pyne *et al.*, 2010; Takuwa, 2002). S1PRs couple to different heterotrimeric G proteins to elicit their functions. S1PR1 couples primarily to G_i, S1PR2 and S1PR3 couple to G_i, G_q and $G_{12/13}$, whereas S1PR4 and S1PR5 couple to G_i and $G_{12/13}$ (Rosen *et al.*, 2009). The differential G protein-coupling of the individual S1PR activates a complex downstream signalling cascade and results in distinct phenotypic changes of the cells (Brinkmann, 2007). S1PR1 and S1PR3 are over-expressed in various types of cancer and contribute to cancer progression *in vitro* and *in vivo* by promoting cell growth, survival, invasion, migration, angiogenesis and chemotherapeutic drug resistance (Balthasar *et al.*, 2006; Bao *et al.*, 2012; Chae *et al.*, 2004; Harris *et al.*, 2012; Herr *et al.*, 2009; Hsu *et al.*, 2012; Kim *et al.*, 2014; Kim *et al.*, 2011; Watson *et al.*, 2010). S1PR2 is generally considered to be anti-tumorigenic (Arikawa *et al.*, 2003; Du *et al.*, 2010; Lepley *et al.*, 2005; Yamaguchi *et al.*, 2003) although its tumour-promoting effects have also been reported (Patmanathan *et al.*, 2016; Ponnusamy *et al.*, 2012; Salas *et al.*, 2011). To date, there are a limited number of studies investigating the roles of S1PR4 and S1PR5 in carcinogenesis. In breast cancer cells, S1PR4 activates the ERK1/2 pathway in a human epidermal growth factor 2 (HER2)-dependent manner (Long *et al.*, 2010b). Upregulation of S1PR5 was found in gliobastoma multiforme (Quint *et al.*, 2014), but it has also been shown that S1PR5 exhibits anti-tumorigenic effects by inducing autophagy in prostate cancer cells (Chang *et al.*, 2009) and inhibiting the proliferation and migration of esophageal cancer cells (Hu *et al.*, 2010). Since opposing functions have been reported for both S1PR2 and S1PR5, their roles could be cancer type-

dependent. It will be important to determine the functional properties of these two receptors to target them effectively.

Re-analyses of two published microarray studies in the present study demonstrated that *S1PR3* were consistently and significantly ($p<0.05$) over-expressed in micro-dissected NPC tumour samples compared to normal nasopharyngeal epithelium in both datasets, while *S1PR2* and *S1PR5* were upregulated in only one of the datasets. These data were in line with previous studies showing the over-expression of these receptors in cancers. For example, over-expression of S1PR2 was detected in oral cancer and glioblastoma multiforme (Bien-Moller *et al.,* 2016; Patmanathan *et al.,* 2016), S1PR3 was upregulated in liver and colon cancers (Wang *et al.,* 2014a), and upregulation of S1PR5 was found in gliobastoma multiforme (Quint *et al.,* 2014). Furthermore, their expression has been negatively correlated with patient survival (Bien-Moller *et al.,* 2016; Quint *et al.,* 2014; Watson *et al.,* 2010). Q-PCR analyses showed that *S1PR2* and *S1PR5* were readily detected in NPC cell lines whereas the expression of *S1PR3* was variable. The role of S1PR2 and S1PR3 in NPC cell migration was investigated by utilising several pharmacological agents, namely a S1PR2 antagonist (JTE-013), a S1PR2 agonist (CYM-5478) and a S1PR1/S1PR3 antagonist (VPC23019). Although the present study did not explore the biological function of S1PR5 due to the limitation of reagents, the involvement of this receptor in S1P-induced migration of NPC cells cannot be ruled out. It remains a possibility that by coupling to G_i, S1PR5 can trigger Rac that lead to an increase in NPC cell migration as suggested by Hu *et al.* (Hu *et al.,* 2010). Coupling to other G proteins such as $G_{12/13}$ might induce an inhibitory effect in the cells (Sugimoto *et al.,* 2003).

7.3.1 S1PR2

Whilst S1PR2 is thought to exhibit anti-migratory effects in general (Arikawa *et al.,* 2003; Lepley *et al.,* 2005; Malchinkhuu *et al.,* 2008; Yamaguchi *et al.,* 2003; Young *et*

al., 2007), there is evidence to show that increased expression of S1PR2 enhances the migration of tumour cells *in vitro* and *in vivo* (Miller *et al.*, 2008; Patmanathan *et al.*, 2016; Ponnusamy *et al.*, 2012; Sekine *et al.*, 2011; Wang *et al.*, 2008). In the present study, addition of JTE-013 suppressed the migration of two NPC cell lines (HONE1 and SUNE1) in the presence of S1P, however, treatment with CYM-5478 did not affect the migration of SUNE1 cells. One possible reason for the inconsistent results is that the suppressive effects of JTE-013 might be due to the inhibition of S1PR4 because JTE-013 is also a S1PR4 antagonist that led to the reduction of ERK1/2 activation in breast cancer cells (Long *et al.*, 2010b). In addition, treatment with JTE-013 has been found to suppress not only S1P-mediated constriction of rat basilar artery, but also constriction induced by prostanoid, endothelin-1, and KCl, and the inhibitory effects of JTE-013 were also observed in mice lacking S1PR2 (Salomone *et al.*, 2008). These data suggest that the decreased S1P-induced migration of NPC cells following JTE-013 treatment might be due to the inhibition of S1PR4. Another possible reason is that JTE-013 is an orthosteric antagonist whereas CYM-5478 is an allosteric agonist, hence the difference in their binding sites might result in coupling to a different subset of G proteins that induce distinct action of S1PR2 in NPC cells (Digby *et al.*, 2010). For instance, coupling of S1PR2 to G_i promotes the migration of esophageal cancer cells (Miller *et al.*, 2008) whereas $G_{12/13}$ coupling inhibits Rac expression and the migration of CHO cells (Sugimoto *et al.*, 2003).

7.3.2 S1PR3

Treatment of two NPC cell lines (HONE1 and SUNE1) with VPC23019 inhibited S1P-induced migration, indicating that S1PR1 and S1PR3 might be involved in the migration of NPC cells. It has been shown that S1P-mediated migration of endothelial cells involved the phosphorylation of S1PR1 at T236 by AKT (Lee *et al.*, 2001). However, the present study focused only on S1PR3 because S1PR3 was the only

receptor whose expression was consistently upregulated in primary NPC in both microarray datasets. To confirm the contribution of S1PR3 to S1P-induced migration of NPC cells, knockdown of S1PR3 was performed using the RNA interference system, a well-recognised option to study the specific role of a candidate molecule (Appasani, 2005). The promoting role of S1PR3 in cell migration has been shown in various types of cancers, including gastric and breast cancer (Kim *et al.*, 2011; Yamashita *et al.*, 2006). In agreement with these observations, the present study showed that siRNA knockdown of S1PR3 reduced the migration of NPC cells in the presence of S1P. Importantly, sensitive RNA *in situ* hybridization (RNAscope) analyses performed by our collaborator, Professor Kwok Wai Lo from The Chinese University of Hong Kong, have confirmed the over-expression of *S1PR3* in primary NPC tissues compared to normal epithelium and epithelium adjacent to the carcinoma (Appendix A). Interestingly, high expression of *S1PR3* significantly correlated with poorer overall survival in NPC patients (Appendix B), data that are similar to those of Watson and co-workers who reported a relationship between S1PR3 expression and patient survival in breast cancer patients (Watson *et al.*, 2010). Taken together, these findings indicated that SPHK1/S1P/S1PR3 signalling plays a crucial role in promoting a migratory phenotype in NPC cells.

7.4 The mechanisms of S1P-induced NPC cell migration

To explore the mechanisms of S1P-induced migration of NPC cells, the involvement of AKT and ERK signalling were examined. There were two reasons for selecting these pathways; firstly, AKT and ERK represent two main downstream targets of the S1P signalling pathway, as they have been shown to be the central players in mediating the oncogenic effects of S1P and their activation promotes cancer cell migration in various types of solid tumours (Kim *et al.*, 2011; Park *et al.*, 2007; Pyne *et al.*, 2010; Van Brocklyn *et al.*, 2003; Yamashita *et al.*, 2006). Secondly, activation of the PI3K/AKT

and MAPK/ERK pathways is a common feature of NPC and these pathways are stimulated by the EBV-encoded LMP1 and LMP2A genes to promote the migration and invasion of NPC cells (Tsao *et al.*, 2015; Wang *et al.*, 2014b). Furthermore, high expression of phosphorylated AKT and ERK in primary NPC is related to lymph node metastasis and radioresistance (Jiang *et al.*, 2014; Yuan *et al.*, 2016).

7.4.1 Activation of AKT and ERK

In the present study, exogenous addition of S1P increased the levels of phospho-AKT and phospho-ERK whereas knockdown of SPHK1 suppressed only the phosphorylation of AKT. The unchanged levels of phospho-ERK following SPHK1 knockdown could possibly be due to the fact that basal levels of phospho-ERK in HONE1 cells were relatively low and, therefore, reduced levels of S1P might not be sufficient to modulate the pathway. Of note, the levels of total AKT and ERK proteins were not altered following the treatment with S1P or SPHK1 knockdown, suggesting that SPHK1/S1P only activates these two proteins but does not regulate their transcription.

7.4.2 S1P/S1PR3 signalling promotes NPC cell migration through the activation of AKT

Focusing on the involvement of AKT signalling in S1P-induced cell migration, NPC cells were treated with a PI3K/AKT inhibitor (LY294002). The treatment significantly reduced the phosphorylation of AKT and suppressed the migration of NPC cells in the presence of S1P. These data were supported by the reduction of phospho-AKT levels in HONE1 cells observed following knockdown of SPHK1 and expression of a constitutively active AKT restored the migration of these cells, suggesting that S1P induced the migration of NPC cells through activation of AKT. These results are in agreement with previous studies showing that S1P activated AKT and promoted the cell

migration of thyroid, ovarian and breast cancers (Balthasar *et al.*, 2006; Kim *et al.*, 2011; Park *et al.*, 2007). Knockdown of S1PR3 inhibited the migration of NPC cells in the presence of S1P and this was accompanied by reduced levels of phospho-AKT. The migration of cells with reduced S1PR3 expression following gene knockdown was partly restored following the expression of a constitutively active AKT. These findings indicated S1PR3 is responsible for the pro-migratory effects of S1P in NPC cells by activating AKT. S1PR1 and S1PR3 are commonly implicated in enhancing cell migration by activating signalling pathways including PI3K/Rac and ERK1/2 (Bergelin *et al.*, 2010; Li *et al.*, 2009a; Long *et al.*, 2010a). The involvement of S1PR3 in mediating S1P-induced cancer cell migration through activation of AKT has only been indirectly inferred previously in a limited number of studies. For example, S1P activated AKT in ovarian and thyroid cancer cells and treatment of these cells with a PI3K/AKT inhibitor (LY294002 or wortmannin) suppressed S1P-induced migration in a G_i-dependent manner (Balthasar *et al.*, 2006; Park *et al.*, 2007). Although these studies revealed the possible involvement of S1PR1 and S1PR3 in mediating these effects by using the S1PR1/3 inhibitor, VPC23019, it was not clear which receptor was precisely responsible. Similar observations were reported in breast cancer cells, but S1P-induced migration was mediated through $G_{\alpha q}$ (Kim *et al.*, 2011). Although knockdown of $G_{\alpha q}$ 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. Thus, the present study is the first to clearly show that S1P induced cancer cell migration by activating AKT via S1PR3.

It has been shown that SPHK1 and S1PR3 function in an amplification loop to promote the migration of breast cancer cells (Long *et al.*, 2010a). S1P was found to stimulate the translocation of SPHK1 to the plasma membrane of breast cancer cells via a S1PR3-dependent mechanism and SPHK1 was in turn required for the expression of

S1PR3 (Long *et al.*, 2010a). The pro-migratory ability of S1P was mediated through S1PR3 by accumulating phosphorylated ERK1/2 into membrane ruffles/lamellipodia and nucleus of the breast cancer cells (Long *et al.*, 2010a). However, in the present study, exogenous S1P induced the phosphorylation of ERK in NPC cells but knockdown of SPHK1 did not affect the levels of phospho-ERK. Furthermore, the expression of S1PR3 remained unchanged following knockdown of SPHK1 in HONE1 cells. Nonetheless, the possibility that S1PR3 can also mediate the S1P-induced migration of NPC cells through mechanisms other than AKT activation cannot be ruled out. S1P/S1PR3 signalling has been shown to promote cancer cell migration/invasion by up-regulating the expression of EGFR, HIF-1α or CRP (Hsu *et al.*, 2012; Kalhori *et al.*, 2013; Kim *et al.*, 2014). The identification of the downstream effector(s) of S1P/S1PR3/AKT signalling in stimulating NPC cell migration warrants further investigation.

7.5 Contribution of EBV infection to the expression of SPHK1 and S1PR3

7.5.1 Establishment of EBV-infected CNE1 and TW04 cells

There is a strong etiological association between EBV infection and NPC, but the exact contribution of EBV to the development of NPC is still largely unclear (Tsao *et al.*, 2015). A major challenge in establishing truly representative NPC cell lines is to develop tumour-derived cell lines that retain the EBV genome, which is commonly lost during prolonged culture (Chang *et al.*, 1989; Glaser *et al.*, 1989; Huang *et al.*, 1980). By using the cell-to-cell contact method, which involves co-cultivation of EBV-negative epithelial cells with Akata cells carrying a recombinant EBV (Imai *et al.*, 1998), several EBV-infected NPC cell lines (CNE2, HK1, HONE1, SUNE1, TW01) and an EBV-infected immortalised nasopharyngeal epithelial cell line (NP460hTert) have been established (Chang *et al.*, 1999; Hau *et al.*, 2011; Lo *et al.*, 2006; Stewart *et al.*, 2004; Tsang *et al.*, 2010). However, the basal levels of SPHK1 and S1PR3 in these

cell lines are readily detected and might not be ideal models to examine whether EBV can modulate the expression of these two molecules. Therefore, in the present study two NPC cell lines (CNE1 and TW04) with low or undetectable levels of SPHK1 and S1PR3 were chosen to generate additional EBV-positive epithelial *in vitro* models. Similar to other EBV (Akata)-infected nasopharyngeal epithelial cell lines, the newly established CNE1/EBV and TW04/EBV cell lines expressed EBNA1 and LMP2A, but not LMP1.

Previous studies have shown that the expression of LMP1 was only detected in EBV-infected nasopharyngeal epithelial cells at the early stages of EBV infection and lost in the later passages (Tsang *et al.*, 2012; Tsang *et al.*, 2010), implying that the expression of LMP1 was required to overcome the cellular stress caused by EBV infection (Hsiao *et al.*, 2009). Although it remains uncertain, the absence of LMP1 might also be a specific characteristic of epithelial cells infected with the EBV Akata strain that was originally isolated from a BL patient. To date, the EBV Akata strain is the only recombinant EBV available for *in vitro* experiments in the EBV/NPC research community. Recently, an EBV strain, M81, isolated from a NPC patient was reported to show a strong tropism to epithelial cells (Tsai *et al.*, 2013) and it could be a more relevant strain to be used to infect epithelial cells *in vitro*.

Consistent with the characteristics of the previously established HK1/EBV cells (a kind gift from Prof Tsao, University of Hong Kong), EBV infection promoted the migration and invasion, but decreased the proliferation of CNE1 and TW04 cells. While EBV infection is well-recognised to enhance NPC cell migration and invasion (Lui *et al.*, 2009; Teramoto *et al.*, 2000; Wu *et al.*, 2003), EBV-infected cells do not acquire proliferative advantage *in vitro* (Tsang *et al.*, 2010; Tsao *et al.*, 2012). The reduced proliferation of EBV-infected cells *in vitro* could possibly be due to the induction of

p16 and p21 expression (Tsang *et al.*, 2012) as well as the lack of tumour microenvironment which consists of molecules that may support the growth of NPC cells following EBV infection (e.g. angiogenic factors and inflammatory cytokines) (Tsao *et al.*, 2012). Indeed, EBV infection has been shown to confer a growth advantage to NPC cells *in vivo* because tumours formed in the athymic nude mice injected with EBV-infected NPC cells grew faster than those with the parental controls (Teramoto *et al.*, 2000). Furthermore, since EBERs have been shown to induce the production of anti-proliferative type-I IFN by activating RIG-1 and TLR3 signalling in BL cells (Iwakiri *et al.*, 2009; Yoneyama *et al.*, 2004), similar mechanisms might occur in NPC cells leading to a reduction in the proliferation of EBV-infected NPC cells *in vitro*.

7.5.2 Expression of SPHK1 and S1PR3

EBV infection contributes to the pathogenesis of NPC by modulating a number of key cellular gene expression programmes (Raab-Traub, 2002). The present study showed that the only EBV-positive NPC cell line, C666-1, expressed the highest levels of SPHK1 and S1PR3 compared to a panel of seven EBV-negative NPC cell lines. Since EBV is present in all NPC cells, and SPHK1 and S1PR3 are over-expressed in primary NPC tissues (Li *et al.*, 2015b) (Appendix A), these observations indicated that EBV infection might regulate the expression of SPHK1 and S1PR3 in NPC.

7.5.2.1 EBV-infected nasopharyngeal epithelial cells

The regulation of SPHK1 expression can be induced in various cell types by several molecules, including platelet-derived growth factor (PDGF), histamine, 12-O-tetradecanoylphorbol-13-acetate (TPA), nerve growth factor (NGF) and IL-1 (Francy *et al.*, 2007; Huwiler *et al.*, 2006; Paugh *et al.*, 2009; Sobue *et al.*, 2005). In addition, a wide variety of stimuli including cytokines, growth factors, GPCR agonists, hormones, phorbol esters, vitamin D_3 and antigens can activate SPHK1 (Pyne *et al.*, 2000). In the

present study, EBV infection increased *SPHK1* mRNA expression as well as the levels of total and phosphorylated SPHK1 proteins in five of eight NPC/immortalised nasopharyngeal cell lines analysed. Although an increase of *SPHK1* mRNA was demonstrated in EBV-infected TW01 cells, the levels of SPHK1 proteins remained unchanged. Since post-transcriptional or post-translational events are known to affect protein expression (Greenbaum *et al.*, 2003), these events might have occurred in the regulation of SPHK1 protein expression in TW01 cells. A study in gastric cancer cells has demonstrated that the expression of SPHK1 can be regulated by lysophosphatidic acid (LPA) through the crosstalk between lysophosphatidic acid receptor 1 (LPAR1) and EGFR (Shida *et al.*, 2008a). Previous work from our laboratory has shown that autotaxin, the enzyme that produces LPA, is highly expressed in NPC (Yap *et al.*, 2015). Furthermore, EBV infection has been shown to increase the expression of autotaxin in Hodgkin lymphoma (Baumforth *et al.*, 2005) and EGFR in cervical cancer (Miller *et al.*, 1995), suggesting that EBV may increase the expression of SPHK1 in NPC through the regulation of LPA and EGFR. Nonetheless, the present study identified a novel role for EBV in stimulating SPHK1 expression and activation in NPC cells.

Unlike SPHK1, EBV infection of nasopharyngeal epithelial cells did not consistently alter the expression of S1PR3, with an increase in three cell lines (HK1, TW04 and NP460hTert) but decrease in another three cell lines (CNE2, SUNE1 and HONE1) and unchanged in two cell lines (CNE1 and TW01). There is a paucity of information on the regulation of S1PR3 expression. A previous study has demonstrated that TGF-β1 can increase the expression of *S1PR3* in a Smad2/3- and Smad4-dependent manner in skeletal myoblasts (Cencetti *et al.*, 2010). Whilst EBV infection has been reported to induce the expression of TGF-β1 in cervical cancer (Cayrol *et al.*, 1995), there are also studies showing that EBV infection can reduce the levels of Smad2 and Smad4 proteins

in Hodgkin lymphoma and gastric cancer (Flavell *et al.*, 2008; Kim *et al.*, 2016). It remains to be explored whether EBV can regulate the expression of S1PR3 through Smad-dependent TGF-β1 signalling in NPC cells.

Of note, the upregulation of SPHK1 and S1PR3 expression was only consistently shown in HK1 cells following EBV infection and their expression appeared to be negatively correlated in the other seven EBV-infected cell lines, suggesting that EBV could regulate the expression of SPHK1 and S1PR3 in a mutually exclusive manner. While this speculation needs to be tested and since all the cell lines analysed express varied levels of SPHK1 and S1PR3, it is unlikely that the endogenous levels of these two genes would determine whether EBV can regulate their expression. Nonetheless, these results revealed a possible role for EBV in modulating the expression of S1PR3 that warrants further investigation.

7.5.2.2 NPC cells transfected with EBV latent genes

EBV exhibits a latency II programme in NPC and the expression of EBV latent genes is restricted to EBNA1, LMP1, LMP2, EBERs, BART miRNAs and BARF1 (Tsao *et al.*, 2015). Having shown that EBV infection can upregulate the expression and activation of SPHK1, HONE1 cells stably transfected with EBNA1, LMP1 or LMP2A were used to determine which of these latent genes was responsible. The results showed that all three EBV latent genes could increase both the mRNA and protein levels of SPHK1 in HONE1 cells. Both LMP1 and LMP2A can activate the MAPK/ERK pathway in NPC, suggesting that these two EBV genes could possibly stimulate the phosphorylation of SPHK1 (Pitson *et al.*, 2003). LMP1 and LMP2A are also well known to promote NPC cell migration (Chen *et al.*, 2014; Fotheringham *et al.*, 2012; Lan *et al.*, 2012; Liu *et al.*, 2012a), via a variety of mechanisms including the upregulation of wingless-type MMTV integration site family member 5A (WNT5A)

which was identified during the course of this study (Yap *et al.*, 2014) (Appendix C). Therefore, the expression of SPHK1 was examined in HK1 cells transiently transfected with LMP1 or LMP2A by Q-PCR. However, the expression of *SPHK1* remained unchanged in HK1 cells transfected with either LMP1 or LMP2A. HONE1 cells were derived from an undifferentiated NPC tumour, whereas HK1 cells established from a well-differentiated squamous cell carcinoma and, therefore, the mechanisms by which EBV regulates SPHK1 expression might be different depending on the genetic background of the cells (Glaser *et al.*, 1989; Huang *et al.*, 1980). In addition, it is possible that the activation of SPHK1 is mediated by other EBV-encoded latent genes. For example, the EBERs can induce the production of IGF-1, a growth factor that has been shown to activate SPHK1 in other cell systems (El-Shewy *et al.*, 2006; Iwakiri *et al.*, 2005).

In the present study, EBV infection was shown to upregulate the expression of S1PR3 in HK1 cells and therefore, the EBV latent gene(s) responsible for this effect was investigated in these cells. However, transient transfection of either LMP1 or LMP2A did not increase the expression of S1PR3 in HK1 cells. It is possible that other EBV-encoded latent genes (e.g. EBNA1, EBERs, BART miRNAs and BARF1) could regulate the expression of S1PR3 independently or jointly. Furthermore, given that EBV-encoded genes can activate multiple signalling pathways, the expression of S1PR3 could be regulated by highly complex mechanisms.

7.6 Limitations of the study

Although this study has been carefully designed to address the specific research questions, there are a number of limitations that could potentially influence the broader conclusions drawn from the data. Careful consideration of such limitations should be given before generalisation and application to practice.

Firstly, both microarray and RNAscope analyses demonstrated the over-expression of *S1PR3* mRNA in primary NPC tissues compared to non-malignant nasopharyngeal epithelium, but post-transcriptional and post-translational modifications might occur to alter the protein expression. Therefore, it would be beneficial to confirm the over-expression of S1PR3 at the protein level in NPC tissues.

Secondly, the analyses of the effects following SPHK1 knockdown are based on the assumption that S1P levels would be reduced. Measurement of S1P levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in conditioned media following SPHK1 knockdown would be helpful to confirm the conclusions.

Thirdly, the interpretations of the findings deduced from the use of pharmacological agents in this study are limited by the assumption that they specifically and effectively modulate their specified target(s). The contradicting data on the roles of S1PR2 could be due to the "off-target" effects of the pharmacological drugs. Therefore, the use of more specific approaches such as ectopic expression or knockdown of S1PR2 would be beneficial to confirm the results.

Lastly, the results of the present study represent the first pre-clinical, proof of concept *in vitro* studies to identify a role for S1P in the pathogenesis of NPC. However, currently the main challenge in NPC research is the lack of truly representative NPC cell lines that are EBV-positive. Therefore, it would obviously be important to determine if these results could be recapitulated using relevant *in vitro* and *in vivo* models.

7.7 Future Work

The results of this study convincingly show that aberrant activation of S1P signalling promotes the migratory phenotype of NPC cells and EBV infection can contribute to the

dysregulation of this signalling pathway. There are a number of areas of research arising from this work that are likely to have fundamental implications for a better understanding of the contribution of S1P signalling to the pathogenesis of NPC and subsequently the development of new strategies to target this pathway therapeutically.

Both microarray and RNAscope analyses showed that the mRNA levels of *S1PR3* are overexpressed in primary NPC tissue samples compared to non-malignant nasopharyngeal epithelium. Currently a suitable anti-S1PR3 antibody for immunohistochemistry is not available for use in NPC tissues. The generation of an antibody suitable for immunohistochemical analyses using paraffin-embedded (FFPE) NPC tissues would be very useful. Examination of the association between S1PR3 protein levels and clinicopathological parameters *in vivo* would be informative and crucial.

In addition to SPHK1 and the S1PRs, it would be informative to evaluate whether the expression of other key regulators of S1P signalling, such as SPHK2, S1P transporters and S1P lyase, is altered in NPC and how their deregulation influences NPC cell behaviour. Collectively, this information will provide a fuller understanding of the contribution of aberrant S1P signalling to the pathogenesis of NPC.

It is now recognised that the tumour microenvironment plays a crucial role in tumorigenesis. In particular, undifferentiated NPC is characterised by a prominent lymphocytic infiltration, pointing to an important role for the immune microenvironment in the pathogenesis of NPC. A comprehensive study of the association of S1P signalling with the NPC tumour microenvironment, including potential crosstalk with immune cells and CAFs, would greatly strengthen our understanding of the contribution of S1P signalling to NPC development and the therapeutic relevance of targeting this pathway.

The present study demonstrated that S1P induced NPC cell migration through the activation of AKT via S1PR3. However, the knockdown of S1PR3 and transfection of a constitutively active AKT only partially reduced/restored the migration of NPC cells, indicating that S1P-mediated migration of NPC cells might involve the activation of other molecules or S1PR. Therefore, exploring more downstream targets of S1P or signalling crosstalk with other oncogenic pathways might provide additional insights into the underlying mechanisms contributing to the oncogenic effects of S1P in NPC.

Having shown *in vitro* that EBV infection contributes to the aberrant activation of S1P signalling, it would be informative to identify the mechanisms employed by EBV to deregulate this pathway and investigate the therapeutic potential of inhibitors or modulators of the S1P signalling pathway *in vivo*. Furthermore, functional studies on the role of S1P in relation to EBV infection in nasopharyngeal epithelial cells, such as EBV infection rate or persistence of EBV, would also be useful.

CHAPTER 8: CONCLUDING REMARKS

The present study reveals for the first time, an oncogenic role of S1P signalling in the pathogenesis of NPC and the contribution of EBV infection to the deregulation of this pathway. In the context of NPC cell migration, a working model can be proposed to demonstrate the link between EBV infection and S1P signalling (Figure 8.1).

The presence of EBV is invariably detected in non-keratinising NPC cells. The present study shows that EBV infection can upregulate the expression and activation of SPHK1 and therefore, high levels of S1P are likely to be present in NPC cells. S1P binds to S1PR3 which was found to be overexpressed in micro-dissected primary NPC tissue samples compared to normal nasopharyngeal epithelium. The SPHK1/S1P/S1PR3 signalling pathway stimulates the phosphorylation of AKT, leading to the migration of NPC cells. The data demonstrate a direct role for S1PR3 in mediating the S1P-induced migration of NPC cells through activation of AKT. These observations are novel and relevant to the field of both NPC pathogenesis and S1P signalling.

From the proposed model, there are three possible ways to abrogate the oncogenic effects of S1P signalling in NPC within a therapeutic context. One option would be to inhibit the activity of SPHK1, which would reduce the amount of S1P produced by the tumour cells. Secondly, S1P itself could be targeted with therapeutic neutralising antibodies and thirdly, specific inhibitors of S1PR3 could be employed to inhibit NPC cell migration and invasion. Taken together, the present study has identified the SPHK1/S1P/S1PR3 axis as a promising target for the development of novel treatment strategies for NPC patients.

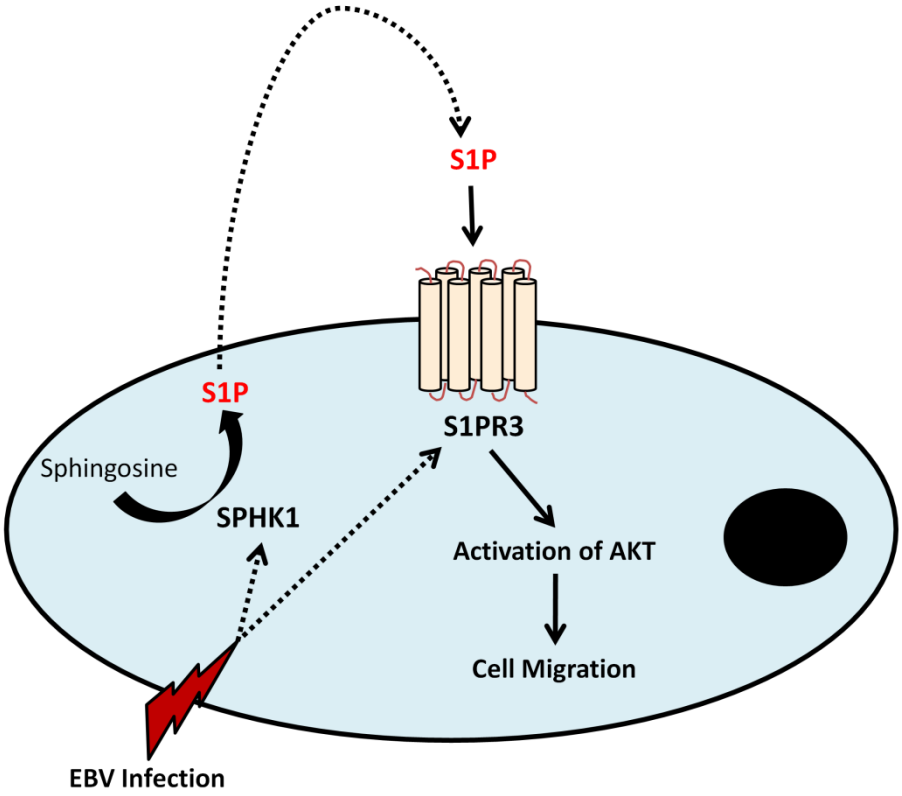


Figure 8.1: A proposed model of S1P-mediated migration of EBV-associated NPC cells

EBV infection can upregulate the levels of SPHK1 and/or S1PR3. High expression of SPHK1 is most likely to result in elevated levels of S1P in NPC cells. S1P can promote the migration of NPC cells by stimulating the phosphorylation of AKT through S1PR3.

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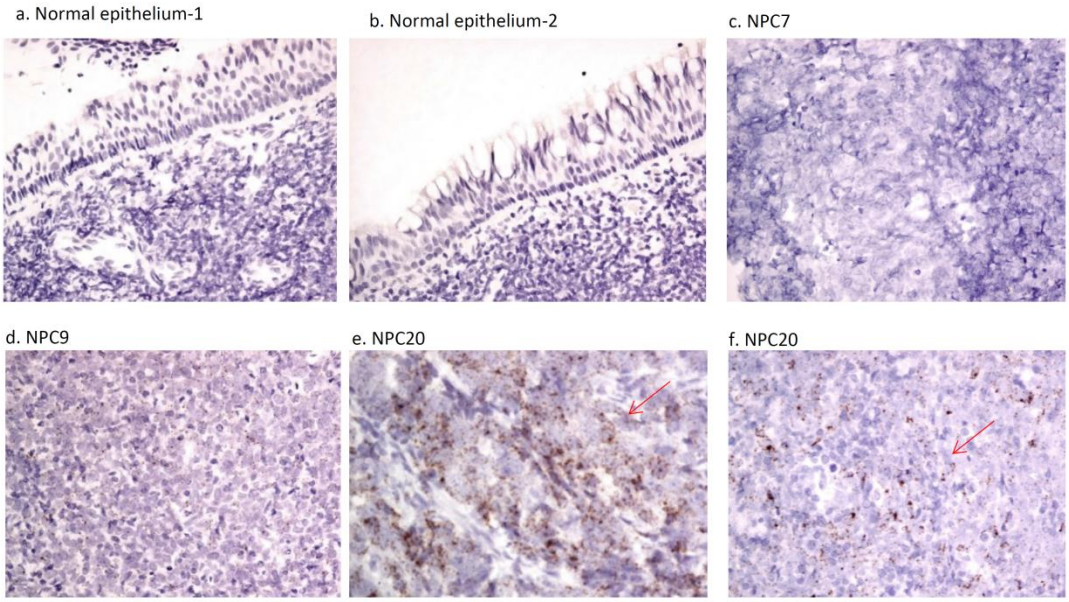
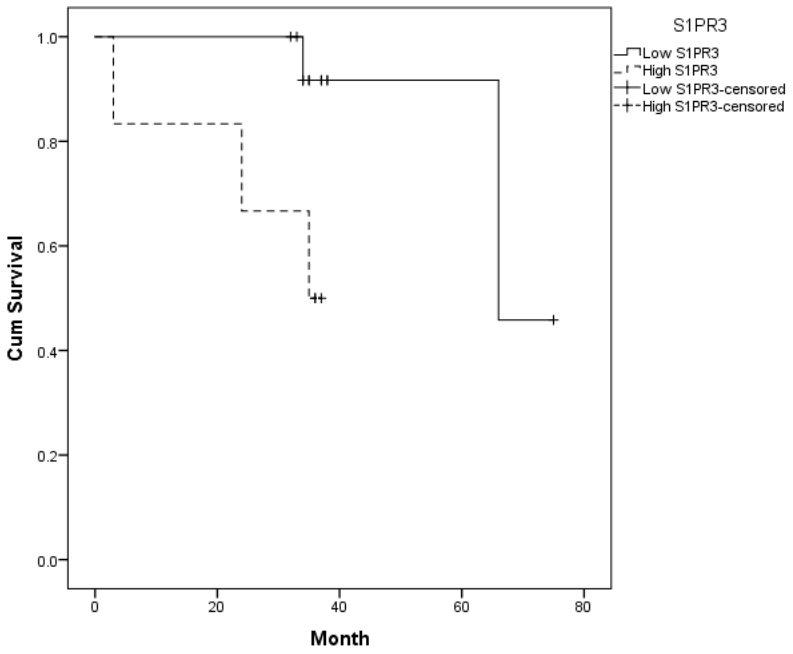
OVER-EXPRESSION OF *S1PR3* IN PRIMARY NPC TISSUES



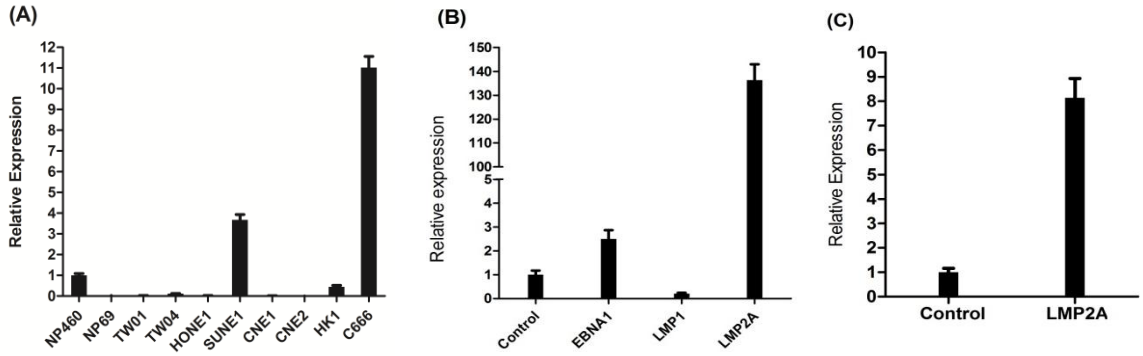
As determined by RNAscope analyses, (a,b) two separate examples of normal epithelium showed negative *S1PR3* staining and representative NPC cases demonstrated (c) negative, (d) weak and (e,f) strong expression of *S1PR3* in the carcinoma (red arrows) (magnification: X400).

REDUCED SURVIVAL IN PATIENTS WITH HIGH *S1PR3* EXPRESSION



Kaplan-Meier survival analysis revealed that high expression of *S1PR3* in NPC was associated with reduced patient survival ($p<0.05$).

EBV INFECTION STIMULATES THE EXPRESSION OF WNT5A



(A) In NPC cell lines, Q-PCR analysis showed that the only EBV-positive cell line, C666-1, had markedly increased levels of WNT5A when compared to a panel of EBV-negative cell lines. Shown here are data comparing cell lines with NP460 cells, an immortalised nasopharyngeal epithelial cell line. (B) Q-PCR analysis showed that the expression of WNT5A transcripts was significantly increased in HONE1 cells expressing EBV-encoded EBNA1 and LMP2A ($p<0.01$). (C) The ability of LMP2A to stimulate the expression of WNT5A was further confirmed in CNE2 cells expressing LMP2A ($p < 0.01$). Data are expressed as the relative expression between the cells transfected with EBV latent genes and their respective controls. The expression levels of the controls were normalised to 1.

LIST OF PUBLICATIONS

1. HM Lee, KW Lo, W Wei, SW Tsao, MH Ibrahim, CW Dawson, PG Murray, IC Paterson and LF Yap. (2016). Oncogenic S1P signalling in EBV-associated nasopharyngeal carcinoma activates AKT and promotes cell migration through S1P receptor 3. Under revision for *Journal of Pathology* (Manuscript ID: 16-654).

2. LF Yap, S Velapasamy, HM Lee, S Thavaraj, R Pathmanathan, W Wei, K Vrzalikova, MH Ibrahim, A Khoo, SW Tsao, IC Paterson, GS Taylor, CW Dawson and PG Murray. (2015). Down-regulation of LPA receptor 5 to aberrant LPA signalling in EBV-associated nasopharyngeal carcinoma. *Journal of Pathology, 235*(3), 456-465.

3. LF Yap, M Ahmad, MM Zabidi, TL Chu, SJ Chai, HM Lee, PV Lim, W Wei, C Dawson, SH Teo and AS Khoo. (2014). Oncogenic effects of WNT5A in Epstein-Barr Virus-associated nasopharyngeal carcinoma. *International Journal of Oncology, 44*(5), 1774- 1780.

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ORIGINAL PAPER

Down-regulation of LPA receptor 5 contributes to aberrant LPA signalling in EBV-associated nasopharyngeal carcinoma

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Abstract

Undifferentiated nasopharyngeal carcinoma (NPC) is a highly metastatic disease that is consistently associated with Epstein–Barr virus (EBV) infection. In this study, we have investigated the contribution of lysophosphatidic acid (LPA) signalling to the pathogenesis of NPC. Here we demonstrate two distinct functional roles for LPA in NPC. First, we show that LPA enhances the migration of NPC cells and second, that it can inhibit the activity of EBV-specific cytotoxic T cells. Focusing on the first of these phenotypes, we show that one of the LPA receptors, LPA receptor 5 (LPAR5), is down-regulated in primary NPC tissues and that this down-regulation promotes the LPA-induced migration of NPC cell lines. Furthermore, we found that EBV infection or ectopic expression of the EBV-encoded LMP2A was sufficient to down-regulate LPAR5 in NPC cell lines. Our data point to a central role for EBV in mediating the oncogenic effects of LPA in NPC and identify LPA signalling as a potential therapeutic target in this disease.

Keywords: nasopharyngeal carcinoma; Epstein–Barr virus; lysophosphatidic acid; LPA receptor

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No conflicts of interest were declared.

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer with high metastatic potential that is particularly prevalent in South East Asia and southern China [1]. Radiotherapy is effective against early-stage NPC; however, over 75% of cases present with late-stage disease and there are significant rates (~30%) of distant metastases subsequent to treatment in these cases [2]. Furthermore, most survivors of NPC have an impaired quality of life due to the location of the tumour at the base of the skull and in close proximity to many vital structures. Unfortunately, our current understanding of the molecular basis of NPC is still inadequate to inform any personalized treatment strategies.

Unlike other head and neck cancers, NPC is consistently associated with Epstein–Barr virus (EBV) infection [3]. EBV latent protein expression in NPC is restricted to Epstein–Barr nuclear antigen (EBNA) 1, latent membrane proteins (LMP1 and LMP2), and BARF1. Although the exact contribution of EBV to the pathogenesis of NPC is still to be elucidated, it is well recognized that EBV alters many functional properties that are involved in tumour progression. NPC characteristically presents with a prominent lymphocyte infiltration, indicating that the tumour microenvironment can influence some of the malignant features of NPC tumour cells. The consistent expression of EBV proteins in NPC cells has led to several clinical trials of adoptive T-cell therapy or vaccination to boost the immune response to these antigens [4]. While there was evidence of a clinical response in some patients, it is not known why other patients did not respond, which may be due to varying degrees of immunosuppression in the tumour microenvironment [5].

Given that distant metastasis remains a major cause of death for NPC patients, we focused on the possibility that lysophosphatidic acid (LPA), a bioactive lipid that

Oncogenic effects of WNT5A in Epstein-Barr virus-associated nasopharyngeal carcinoma

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Abstract. The molecular events that drive the progression of Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC) are still to be elucidated. Here, we report for the first time the pathogenic significance of an NPC-associated gene, wingless-type MMTV integration site family, member 5A (WNT5A) and the contribution of EBV to its expression. WNT5A is a representative Wnt protein that activates non-canonical Wnt signalling. With regard to its role in carcinogenesis, there is conflicting evidence as to whether WNT5A has a tumour-promoting or tumour-suppressive role. We show that WNT5A is upregulated in primary NPC tissue samples. We also demonstrate that WNT5A expression was dramatically increased in NPC cell lines expressing the EBV-encoded LMP2A gene, suggesting that this EBV-encoded latent gene is responsible for upregulating WNT5A in NPC. In addition, *in vitro* WNT5A overexpression promotes the proliferation, migration and invasion of NPC cells. Our results not only reveal pro-tumorigenic effects of WNT5A in NPC but also suggest that WNT5A could be an important therapeutic target in patients with EBV-associated disease.

Introduction

Nasopharyngeal carcinoma (NPC) is particularly prevalent in southern China and Southeast Asia (1). Radiotherapy is effective against early stage NPC; however, over 70% of cases present with late stage diseases and only 10-40% of these patients survive more than 5 years (2-4). Currently the mainstay of treatment for locoregional advanced cases of NPC is concurrent chemoradiotherapy. Unfortunately, undesirable complications frequently occur after treatment because of the location of the tumour at the base of the skull, closely surrounded by, and in close proximity to, many vital structures that result in high morbidity and poor quality of life (5). Unlike other head and neck cancers, NPC is consistently associated with Epstein-Barr virus (EBV) infection (6). EBV latent gene expression in NPC is restricted to EBNA1, BARF1, variable expression of LMP1 and LMP2A, and consistent expression of the non-coding EBER1/2 RNAs and BARTs, a family of viral microRNAs. The molecular events that drive the progression of NPC, including the exact contribution of EBV to the pathogenesis of NPC, are still to be elucidated.

The Wnt signalling pathways have historically been divided into two classes: namely the canonical and non-canonical pathways. The canonical signalling pathway induces the nuclear accumulation and transcriptional activation of β-catenin. It is the most intensively studied Wnt pathway implicated in cancer development by promoting cancer cell proliferation and migration (7). By contrast, the non-canonical pathway is essentially an umbrella term for all Wnt-activated cellular signalling pathways that do not promote β-catenin-mediated transcription. The non-canonical and planar cell polarity (PCP) pathways promote calcium mobilization and activate downstream pathways involved in cell motility and metastasis. However, emerging evidence suggests that these pathways are not as autonomous as originally thought and there may be cross-talk between these two pathways (8). WNT5A is a representative of Wnt protein that activates non-canonical Wnt signalling, it can, under certain circumstances, signal through the canonical pathway (8). A number of studies indicates that WNT5A has a tumour-suppressing effect with reduced expression being reported in colorectal cancer (9,10), neuroblastoma (11), ductal breast cancer (12,13) and leukemias (14-16). Conversely,

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LIST OF PRESENTATIONS

1. HM Lee, CW Dawson, PG Murray, IC Paterson, LF Yap. Sphingosine-1-phosphate promotes cell motility in Epstein-Barr virus-associated nasopharyngeal carcinoma through activation of AKT via S1P receptor 3. Presented: 5th NPC Research Day, University of Malaya, Kuala Lumpur (2016). Awarded the best oral presentation.

2. HM Lee, CW Dawson, PG Murray, IC Paterson, LF Yap. Aberrant activation of sphingosine-1-phosphate signalling promotes migration of Epstein-Barr virus-associated nasopharyngeal carcinoma cells. Presented: Frontiers in Cancer Science 2015, National University of Singapore, Singapore (2015).

3. HM Lee. Aberrant Sphingosine-1-phosphate signalling in nasopharyngeal carcinoma. Presented: PhD Candidature Defence, University of Malaya, Kuala Lumpur (2015).

4. HM Lee. S1P – A driver of NPC. Presented: Three Minute Thesis 2015 Competition, University of Malaya, Kuala Lumpur (2015).

5. HM Lee, CW Dawson, PG Murray, IC Paterson, LF Yap. Oncogenic effects of sphingosine-1-phosphate signalling in EBV-associated nasopharyngeal carcinoma. Presented: 4th NPC Research Day, University of Malaya, Kuala Lumpur (2015). Awarded the best oral presentation.

6. HM Lee, CW Dawson, PG Murray, IC Paterson, LF Yap. Aberrant sphingosine-1-phosphate signalling in EBV-associated nasopharyngeal carcinoma. Presented: 3rd NPC Research Day, Institute of Medical Research, Kuala Lumpur (2014).

7. LF Yap, CW Dawson, R Pathmanathan, HM Lee, P Lim, T Haigh, JR Arrand, GS Taylor, IC Paterson, PG Murray. Aberrant phospholipid signalling in EBV-

associated nasopharyngeal carcinoma. Presented: American Association of Cancer Research 104th Annual Meeting, Washington DC (2013).

8. HM Lee, CW Dawson, PG Murray, IC Paterson, LF Yap. Aberrant sphingosine-1-phosphate signalling in nasopharyngeal carcinoma. Presented: 2nd NPC Research Day, University of Malaya, Kuala Lumpur (2013).

9. HM Lee. Aberrant sphingosine-1-phosphate signalling in nasopharyngeal carcinoma. Presented: PhD Proposal Seminar, University of Malaya, Kuala Lumpur (2013).