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 the 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 secondly that it can inhibit the activity of EBV-specific cytotoxic T cells. Focussing 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 gene 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
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]. Further, most survivors of NPC have 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 the 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 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 focussed on the possibility that lysophosphatidic acid (LPA), a bioactive lipid that has been shown to
function as a motility factor for cancer cells, could be involved in the pathogenesis of NPC. Emerging evidence suggests that the cancer promoting effects of LPA are mainly a consequence of alterations in the expression or function of one or more of six known G protein-coupled receptors (GPCRs), LPAR1-6 [6]. We were particularly interested to explore if this was also the case in NPC and we show that loss of LPAR5 contributes to the aberrant activation of LPA signalling in NPC and that EBV-encoded oncogene LMP2A down-regulates LPAR5.

Materials and Methods

Cell lines and tissue samples

The cell lines used in this study included two immortalized nasopharyngeal epithelial cell lines, NP69 and NP460; eight NPC-derived cell lines, of which seven are EBV-negative (CNE1, CNE2, HK1, HONE1, SUNE1, TW01, TW04) and one of which is EBV-positive (C666-1); AdAH, a cell line derived from an adenocarcinoma of the nasopharynx; and AGS, a gastric cancer-derived cell line. Cells stably infected with a recombinant EBV (Akata strain) or expressing individual EBV-encoded latent genes were generated as previously described [7-9]. To generate HK1 cells stably expressing LMP2A, the LPM2A fragment was excised from a pSG5/LMP2A plasmid (kindly provided by Prof Richard Longnecker from the Northwestern University Feinberg School of Medicine, Chicago, USA) and sub-cloned into a retroviral vector, pBabe. The retroviral constructs, pBabe-LMP2A and pBabe alone, were transfected into a viral packaging cell line, Phoenix Ampho, using Lipofectamine 2000 (Invitrogen). The viral supernatant was used to transduce HK1 cells in the presence of polybrene and selected in puromycin. Expression of LMP2A protein was confirmed by western blot analysis (Figure S1).
Paraffin-embedded archival NPC tissue samples were collected from the Sime Darby Medical Centre Subang Jaya, Malaysia. All samples were non-keratinising EBER-positive NPC. Ethical approval for this study was obtained from the Independent Ethics Committee, Sime Darby Healthcare, Malaysia (Ref # 201206.2).

**Immunohistochemistry and Immunocytochemistry**

Expression of LPAR5 proteins in primary NPC tissue samples was determined by immunohistochemistry using the DAKO REAL™ EnVision Detection System (DakoCytomation, Denmark). Anti-LPAR5 rabbit polyclonal antibody (Sigma) was used at 1:45. Non-neoplastic tonsils demonstrating reactive lymphoid hyperplasia and omission of the primary antibody were used as positive and negative controls, respectively. Immunohistochemical staining was semi-quantitatively evaluated using the H-score method. The percentage of tumour corresponding to an ordinal intensity value (0 = none, 1 = weak, 2 = moderate, 3 = strong) was assigned using whole sections. The H-score was defined as the sum of the percent of tumour cells staining multiplied by the intensity level, resulting in a score ranging from 0 (no staining in any of the tumor cells) to 300 (strong staining in all tumour cells). Where available, H-scoring was also undertaken for intra-sectional normal surface respiratory epithelium. Immunohistochemistry for autotaxin (ATX) and LMP2A was performed as described previously [10, 11]. For cell staining, cells were seeded on Shandon Multi-Spot slides (Thermo Fisher Scientific) one day prior to staining to achieve 70-80% confluency. Cells were fixed with 4% formaldehyde and endogenous peroxidase activity quenched using DAKO Peroxidase Blocking reagents. Antigen retrieval was performed by immersing the slides in buffer containing 1 mM EDTA/0.05% Tween 20 for 16 hours at 65°C with slight agitation. Cells were then incubated in 5x casein solution for 1 hour, followed by
anti-LPAR5 rabbit polyclonal antibody (1:200; Sigma) for 2 hours. Detection of LPAR5 protein was performed using ImmPRESS HRP/ImmPACT DAB reagents (Vector Laboratories, USA).

**Quantitative real-time PCR (Q-PCR)**

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, UK) and subjected to reverse transcription using oligo(dT) primer and Superscript III (Invitrogen, USA). Q-PCR was performed in triplicate using the ABI Prism 7000 Sequence Detection System and TaqMan Gene Expression Assays (LPAR5: Hs 00252675_s1; ATX: Hs 00905125_m1; Applied Biosystems, USA). GAPDH was amplified in the same reaction to serve as an internal control for normalization. Fold changes in gene expression were measured using the comparative threshold cycle method (ΔΔCt).

**Transwell migration and invasion assays**

Migration assays were carried out using fibronectin-coated (10 µg/ml) polycarbonate filters (8 µm pore size, Transwell, Corning). Cells were incubated in RPMI/0.5% FBS overnight and re-suspended in migration buffer [RPMI containing 0.25 mg/ml fatty acid-free human albumin (Sigma)]. 1 x 10⁶ cells were plated into the upper chamber and allowed to migrate for 19 hours in the presence or absence of 2.5 µM LPA (Enzo Life Sciences) in the lower chamber. Migrated cells were stained with 0.1% crystal violet and counted in five random fields. For experiments using a protein kinase A (PKA) inhibitor (6-22 Amide; Calbiochem), the inhibitor (30 µM) was added to the upper chamber together with the cells and LPA (2.5 µM) added to the lower chamber. For invasion assays, pre-coated invasion chambers (8 µm pore size, BD BioCoat Matrigel) were used and cells allowed to invade for 48 hours.
Interferon-\(\gamma\) release ELISA assays

T cell clones specific for EBV antigens were generated as previously described [12] and epitope peptides synthesised by 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, UK). To avoid the complication caused by the endogenous levels of LPA which are generated by lymphoblastoid cell lines (LCLs), the T cell clones act as both target and responder cells in this assay. T cell clones were seeded in 96-well plates and pulsed with limiting concentrations (0.1 \(\mu\)M – 0.1 pM) of epitope peptide for 4 hours prior to the addition of different concentrations of LPA. The supernatant medium was harvested after 18 hours and the secreted interferon-gamma (IFN-\(\gamma\)) measured by ELISA (Endogen).

LPAR5 overexpression and knockdown

The vector carrying the coding region of LPAR5, pcDNA3.1/LPAR5, was kindly provided by Dr Gabor Tigyi (University of Tennessee Health Science Center, Memphis, USA). The LPAR5 cDNA was sub-cloned into a lentiviral vector, pLVX (Clontech). Briefly, the lentiviral construct (pLVX/LPAR5 or pLVX alone) was transfected into a lentiviral packaging cell line, 293T, using polyethylenimine together with the packaging plasmid (psPAX2) and envelope plasmid (pMDG2). At 48 hour post-transfection, the lentivirus-containing supernatant was centrifuged and filtered through a 0.45 \(\mu\)m syringe filter. The viral supernatant and 8 \(\mu\)g/ml polybrene were then added to plates cultured with CNE1 or TW04 cells and incubated for 16 hours. The virus-containing media was then removed and the cells were cultured in RPMI/10% FBS for an additional 48 hours before selecting the transfected cells with 0.5-1 \(\mu\)g/ml puromycin. To knockdown LPAR5, two pre-designed LPAR5 shRNA lentiviral plasmids (MISSION shRNA Plasmid DNA, Sigma) were used, together with control (random) shRNA. The lentivirus-containing supernatant was collected using the transduction protocol as above. CNE1/pLVX or CNE1/LPAR5 cells were incubated...
with the viral supernatant plus 8 µg/ml polybrene for 16 hours. The virus-containing media was then removed and the cells were cultured in RPMI/10% FBS for additional 8 hours. Cells were then incubated in RPMI/0.5% FBS overnight prior to the Transwell migration assays.

The sequences of the LPAR5 shRNAs are

CCGGACCCGCTGGTGTACTACTTTACTCGAGTAAAGTAGTACACCAGCGGTTTTTTG (NM_020400.5-1319s21c1) and

CCGGGCTCACCACTGAGGCCTAATACTCGAGTATTAGGCCTCAGTGGTGAGCTTTTTG (NM_020400.5-2049s21c1).

Statistical analysis

For Q-PCR and migration/invasion assays, statistical differences between experimental groups were evaluated by Student’s t-test. P values below 0.05 were regarded as significant.

Results

**LPA enhances the migration of NPC cells and inhibits the activation of EBV-specific T cells**

To investigate the phenotypic impact of LPA on NPC cells in vitro, we used two representative NPC cell lines (HK1 and CNE1), as well as AdAH, a cell line derived from an adenocarcinoma of the nasopharynx. LPA enhanced the migration of all three cell lines in transwell assays (Figure 1; p<0.05) without affecting proliferation (data not shown). We next investigated the effect of LPA on the activity of EBV-specific cytotoxic T cells using in vitro cultivated CD8+ T cell clones which recognise epitopes within EBNA1 and LMP2A proteins, both of which are expressed in NPC. We observed a characteristic dose effect of LPA, with maximal T cell inhibition, as observed by reduced IFN-γ release, occurring with low levels of
LPA (1-2.5 μM; Figure 2A-D). Because EBNA1 can also be recognised by CD4+ T cells [13], we repeated the experiment using an EBNA1-specific CD4+ T cell clone. We observed that the exogenous addition of LPA also reduced IFN-γ release from these EBNA1-specific CD4+ T cells (Figure 2E). We conclude that LPA not only enhances the migration of NPC cells, but also suppresses the activity of CTLs specific for EBV latent antigens known to be expressed in NPC tumour cells.

**Expression of autotaxin in NPC**

Having shown the phenotypic impact of LPA on NPC cells *in vitro*, we then investigated the expression of autotaxin (ATX), the key enzyme that produces LPA. Our own previously published microarray data [14] showed that ATX mRNA was significantly up-regulated in 7/15 (46%) cases of micro-dissected NPC cells compared to four cases of normal epithelium (Figure 3A). Of the eight tumours in this series that were available for Q-PCR analysis, five of them showed higher levels of ATX than four normal controls (Figure 3B). A further 20 cases were stained using an ATX-specific monoclonal antibody [10]. Six of these cases showed immunoreactivity for ATX. In most cases staining was in scattered tumour cells as well as dendritic cells and was cytoplasmic and occasionally granular as has previously been described [10] (Figure 3C).

**Down-regulation of LPAR5 expression in NPC**

We opted to explore the mechanisms underpinning the effect of LPA on the migration of NPC cells, focussing on the possibility that alterations in LPA receptor expression might be responsible. A re-examination of our previous microarray data [14] revealed significant down-regulation of LPAR5 mRNA expression in micro-dissected NPC cells compared to
normal epithelium (p<0.0001; Figure 4A). Expression of the other five LPA receptor genes was not significantly different (Table S1). The expression and cellular localization of LPAR5 protein was then examined in an independent cohort of 25 formalin-fixed paraffin-embedded primary NPC and 2 non-malignant nasopharyngeal samples using immunohistochemistry. After first validating the specificity of an LPAR5 antibody (Figure S2), we showed that while normal surface epithelium, including non-dysplastic epithelium adjacent to carcinoma consistently demonstrated strong staining for LPAR5 (mean H-score of 258, range 130-300, mode 300; Figure 4B), LPAR5 was down-regulated in NPC (mean H-score of 51, range 0-200, mode 0, p > 0.0005 independent T-test). An H-score of 0 was present in 13 of 25 (52%) tumours (Figure 4B).

**LPAR5 inhibits NPC cell migration**

Having shown that LPA can increase the migration of NPC cells, we next investigated if this effect was mediated through the down-regulation of LPAR5. We first compared LPAR5 mRNA expression in eight NPC cell lines with that in the immortalized nasopharyngeal epithelial cell lines, NP69 and NP460. The expression of LPAR5 in NP69 and NP460 was similar (p=0.06), while all NPC cell lines showed substantially reduced levels of LPAR5 (p<0.0001; Figure 5A). The protein expression of LPAR5 was examined using immunocytochemical analysis. In agreement with the Q-PCR data, we observed membranous expression of LPAR5 protein in NP69 cells. In contrast, LPAR5 expression was either weak or undetectable in two representative cell lines, CNE1 and TW04 (Figure 5B). We selected these two cell lines for further study because they retained the ability to migrate in response to LPA under serum-free conditions (data not shown). To investigate the functional role of LPAR5, these cell lines were stably transduced with pLVX/LPAR5 or with the empty vector. Increased expression of LPAR5 mRNA in transfected CNE1 and TW04 cells was confirmed
by Q-PCR analysis (Figure 5C). LPA-induced migration was significantly reduced in both CNE1 and TW04 cells over-expressing LPAR5 (Figure 5D). To further confirm the specificity of these effects, we performed rescue experiments by knocking down LPAR5 in transfected CNE1 cells. Q-PCR analysis confirmed the reduced levels of LPAR5 in the transfected cells (Figure S3). Knockdown of LPAR5 in these cells using two independent shRNAs resulted in a significant increase in migration (Figure 5E). Finally, given that LPAR5 has been shown to inhibit cell migration in melanoma cells through activation of the cAMP/PKA pathway [15], we investigated if the anti-migratory effects of LPAR5 were also mediated by activation of this pathway in NPC cells. Treatment of LPAR5-overexpressing NPC cells with a PKA inhibitor (PKAi) counteracted the repressive effects of LPAR5 on cell migration to levels comparable to those observed in untreated vector control cells (Figure 5F). We conclude that down-regulation of LPAR5 promotes LPA-mediated migration of NPC cells and that this effect appears to be mediated through PKA signalling.

**EBV infection of NPC cells mediates the down-regulation of LPAR5 expression**

We next explored the mechanism of LPAR5 down-regulation. In the analysis of LPAR5 expression in the NPC cell lines described above, we had noted that LPAR5 levels were the lowest in EBV-positive C666-1 cells compared to all the other NPC cell lines which are EBV-negative. This prompted us to explore if EBV infection could modulate LPAR5 expression in NPC cells. We stably infected five NPC cell lines (CNE1, CNE2, SUNE1, HK1, HONE1) as well as two other epithelial cancer cell lines (AdAH, AGS) using an Akata-derived recombinant EBV. We found that LPAR5 expression was significantly down-regulated following EBV infection in all NPC cell lines examined and in both AdAH and AGS cells (p<0.002; Figure 6A). The reduced expression of LPAR5 protein was further confirmed in EBV-infected HONE1 cells (Figure 6B).
The EBV-encoded LMP2A gene down-regulates LPAR5 expression in NPC cells

Having shown that EBV infection can reduce LPAR5 levels in NPC cells, we wanted to establish which EBV gene was responsible for this effect by examining LPAR5 expression in HONE1 cells transfected with either LMP1 or LMP2A, both of which have been shown to promote the migration of NPC cells in vitro. We found that LMP2A expression induced a robust down-regulation of LPAR5 mRNA levels in HONE1 cells (p<0.001) while LPAR5 levels remained unchanged in the LMP1-transfected cells (Figure 7A). Similarly, immunocytochemical analysis confirmed the decreased expression of LPAR5 protein in HONE1 cells expressing LMP2A (Figure 7B). We further confirmed that LMP2A reduced LPAR5 expression in two additional NPC cell lines (CNE2 and HK1) (p<0.05; Figure 7C). We conclude that LMP2A, but not LMP1, contributes to the EBV-induced down-regulation of LPAR5 observed in NPC cells. We explored the possibility that LMP2A might regulate LPAR5 expression through one or more of the pathways known to be activated by this viral protein. However, treatment of two LMP2A-expressing NPC cell lines with inhibitors of JNK, p38, PI3K/mTOR and Notch signalling failed to consistently change LPAR5 expression (data not shown). Finally, paraffin sections of 11 of the tumours described above were available and tested for LMP2A using immunohistochemistry. Four tumours tested positive for LMP2A, three of which also showed down-regulation of LPAR5. However, LPAR5 was also down-regulated in all seven tumours which were LMP2A-negative.
Discussion

There is now compelling evidence to show that LPA signalling can affect many key processes involved in carcinogenesis. Although the enzymes that generate or degrade LPA, and even LPA itself, are all potential therapeutic targets in cancer [16], there is a growing appreciation that the oncogenic effects of LPA are probably dependent upon alterations in the biological response of cells to LPA [6]. Among the six well characterised receptors, LPAR2 and LPAR3 are generally regarded as potentially oncogenic because they promote cancer cell motility and metastasis [17]. In contrast, LPAR4 and LPAR5 might act as tumour suppressors, although their biological activities are in general poorly understood [15, 18]. In the present study, we show that LPAR5 is down-regulated in NPC and that this down-regulation promotes LPA-induced migration of NPC cells. Further, LPAR5 also inhibited NPC cell invasion in vitro (Figure S4).

The signalling properties of LPAR5 have been inferred from a limited number of studies. For example, LPA induced a Rho kinase-dependent cytoskeletal contraction in LPAR5-transfected neuroblastoma cells by coupling to \( \text{G}_{\alpha12/13} \) and increased intracellular calcium levels by activation of \( \text{G}_{\alphaq} \) [19]. LPA also increased the levels of cAMP in LPAR5-expressing neuroblastoma and HeLa cells [19, 20]. In B16 melanoma cells, where LPAR5 is the predominant receptor, LPA inhibition of migration was mediated specifically by LPAR5 through the elevation of cAMP [15] and the subsequent activation of PKA. In the present study, we demonstrated an increase in LPA-induced migration of LPAR5-expressing NPC cells following treatment with a PKA inhibitor, implying that the inhibitory effects of LPAR5 in NPC cell migration are similarly mediated through the cAMP/PKA pathway.
The strong etiological link between EBV infection and NPC is well recognized [3]. In the present study, we have shown that EBV infection down-regulates expression of LPAR5 in a number of epithelial cell lines, including five NPC cell lines. These data are consistent with the observation that EBV infection of B cells abrogates the activation of adenylyl cyclase and thereby cAMP formation [21]. Further, we found that EBV-encoded oncogene LMP2A can down-regulate LPAR5 expression in three EBV-infected cell lines. LMP2A mRNA is detectable in NPC and when expressed in certain immortalized epithelial cell lines, it can induce anchorage-independent growth, enhance cell adhesion and cell motility, and inhibit epithelial cell differentiation [22]. We suspected that LMP2A might down-regulate LPAR5 through a pathway known to be activated by LMP2A. However, treatment of LMP2A-expressing cells with inhibitors of JNK, p38, PI3K/mTOR and Notch signalling, failed to consistently change LPAR5 expression, suggesting that LMP2A regulates LPAR5 through a novel pathway. Furthermore, we did not observe a correlation between LPAR5 and LMP2A expression in the series of tumours examined here. There are several explanations for this: First, the sample size is too small, requiring analysis of more samples. Secondly, LMP2A-independent mechanisms may also contribute to the observed reduction in LPAR5 expression. This possibility is supported by our observation that sensitive Q-PCR assays could only detect low levels of LMP2A mRNA expression in C666-1 cells in which we had observed a robust down-regulation of LPAR5. A third possibility is that a proportion of the tumours which tested negative for LMP2A simply expressed levels too low to be detected in our immunohistochemical assay. In keeping with this we have previously observed that LMP2A mRNA is detectable in a much higher proportion of NPC (10 out of 15; 66%; unpublished data). It is also worthy of note that our preliminary studies have also revealed down-regulation of LPAR5 expression in Hodgkin’s lymphoma (HL), another EBV-associated cancer that expresses LMP2A (Figure S5). The involvement of EBV infection in
LPA signalling is interesting because we previously showed that in HL, the expression of autotaxin was induced by EBV infection which in turn led to increased LPA production and tumour cell survival [10]. Although we did not observe any increase in autotaxin following the infection of NPC cells with EBV (data not shown), we showed that autotaxin was expressed in a subset of NPC.

EBV-associated tumours, such as NPC, that arise in overtly immunocompetent individuals must have evolved strategies to avoid immune surveillance. For example, NPC tumour infiltrating lymphocytes were found to contain functionally impaired precursors of anti-LMP1 and -LMP2 cytotoxic T-lymphocytes (CTLs) [23]. Although it is generally recognized that several immunosuppressive factors are released by malignant cells in the tumour microenvironment [5], so far there is no consensus on any major immunosuppressive factors in NPC tumours. Here we show that LPA inhibits the function of EBV-specific T cell clones in vitro, suggesting LPA might be an immunosuppressive factor in the NPC tumour microenvironment. Indeed, a recent report has shown that LPA can suppress T cell receptor signalling and the activation and proliferation of CD8 T cells in mice, leading to impaired tumour growth in vivo [24]. The immunosuppressive effect of LPA in these T cells was mediated by LPAR5. We have also shown that the EBV-specific T cell clones reported in the present study express only LPAR2 and LPAR5 (data not shown). The role of LPAR5 in LPA-induced inhibition of EBV-specific T cell function warrants further investigation.

In conclusion, we report for the first time that LPA signalling is de-regulated in EBV-associated NPC. Therapeutic targeting of LPA signalling in NPC patients might not only inhibit tumour cell migration and metastasis but could also enhance the effectiveness of adoptive EBV-specific CTL or vaccine-based therapies.
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Author contributions

LFY and PGM conceived and designed the study; LFY, SV, HML, KV, MI performed experiments; ST and PR provided clinical materials and analysed immunohistochemical staining; WW performed microarray data analyses; ASK, SWT and CWD produced transfected cell lines; GST supervised the T cell experiments; ICP, GST and CWD critically reviewed the manuscript and contributed intellectual opinion; LFY and PGM wrote the manuscript. LFY and ICP supervised the study.

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Figure legends

**Figure 1.** LPA promotes NPC cell migration. HK1, CNE1 and AdAH cells were allowed to migrate through fibronectin-coated membranes in the absence or presence of 2.5 μM LPA. Data are expressed as mean percentage of cells migrating ± s.d. and the results are expressed relative to migration of cells in the absence of LPA (=100%). LPA significantly enhanced the migration of all three cell lines (p<0.05).
Figure 2. LPA inhibits EBV-specific T cell activity. T cell clones specific for EBV-encoded proteins were seeded in 96-well plates and pulsed with limiting concentrations of epitope peptides for 4 hours prior to the addition of different concentrations of LPA. The supernatant was harvested after 18 hours and the secreted IFN-γ measured by ELISA. The subtypes of the T cells and peptides used for the EBV-encoded proteins are (A) CD8+/CLG[LMP2A], (B) CD8+/IEN[LMP2A], (C) CD8+/LLW[LMP2A], (D) CD8+/HPV[EBNA1], (E) CD4+/VYG[EBNA1]. A range of T cell specificities and subtypes are inhibited by LPA, with maximal inhibition occurring with 1-2.5 μM LPA.
Figure 3. Expression of ATX in NPC. (A) Our previous microarray data showed that ATX mRNA was up-regulated in 46% (7/15) cases of micro-dissected NPC cells (NPC 1-15) compared to normal epithelium (Control 1-4). (B) Up-regulation of ATX mRNA was validated in 5/8 NPC cases by Q-PCR analysis. (C) Immunohistochemical analysis of ATX expression in primary NPC tissues. A representative section of NPC showing positive ATX staining.
Figure 4. Down-regulation of LPAR5 expression in NPC. (A) Our previous microarray data showed that LPAR5 mRNA was significantly down-regulated in micro-dissected NPC cells compared to normal epithelium (Control 1-4; p<0.0001). (B) Immunohistochemical evaluation of LPAR5 expression in primary NPC tissues. a, b; Two separate NPCs demonstrating positive LPAR5 staining. There is less intense staining in the carcinoma (arrow) compared to overlying normal epithelium (asterisk); c, high-power view of area highlighted by arrow in b; original magnification a, x20; b, x100; c, x400. d & e, Two separate examples of negative cases. There is no staining in the carcinoma (arrows) compared to normal overlying epithelium (asterisk); f, high-power view of area highlighted by arrow in e; original magnification d, x20; e, x100, f, x400.
Figure 5. LPAR5 inhibits cell migration in a PKA-dependent manner. (A) Q-PCR analysis of LPAR5 mRNA expression in NPC cell lines. Compared to the two immortalised nasopharyngeal epithelial cell lines (NP69 and NP460), LPAR5 levels were significantly reduced in all eight NPC cell lines examined (p<0.0001). (B) Immunocytochemical analysis showed membranous expression of LPAR5 protein in NP69 cells compared to two NPC cell lines, TWO4 and CNE1. (C) CNE1 and TW04 cells stably transduced with pLVX/LPAR5 (CNE1/LPAR5, TW04/LPAR5) or with vector alone (CNE1/vector, TW04/vector). Q-PCR analysis confirmed the increased expression of LPAR5 in CNE1/LPAR5 and TW04/LPAR5 cells (p<0.001). Vector control cells were normalised to 1. (D) Migration of LPAR5-expressing CNE1 and TW04 cells in fibronectin-coated transwell assays in the presence of LPA (2.5 μM) in the lower chamber. (E) Transwell assays of transient LPAR5 knock-down in CNE1/LPAR5 and CNE1/vector cells using two shRNA plasmids (shRNA1 & shRNA2), together with control shRNA (NS). (F) Transwell migration assays of CNE1/LPAR5, TW04/LPAR5 and their respective control cells in the presence of LPA (2.5 μM) in the lower chamber and a PKA inhibitor (6-22 Amide; 30 μM) in the upper chamber. Data for transwell migration assays are expressed as mean percentage of cells migrating ± s.d. expressed relative to migration of vector control cells (=100%). * p<0.05; ** p<0.01; *** p<0.001.
Figure 6. Down-regulation of LPAR5 following EBV infection. (A) Q-PCR analysis of LPAR5 levels in five NPC cell lines (CNE1, CNE2, SUNE1, HK1 and HONE1) and two epithelial cell lines (AdAH and AGS) stably infected with a recombinant EBV (Akata strain). Data are expressed as the relative expression between the cells infected with EBV and their respective controls (normalised to 1). ** p<0.01; *** p<0.001. (B) Immunocytochemical analysis of LPAR5 in HONE1 cells following EBV infection. NP69 cells were used as a positive control.
Figure 7. EBV-encoded LMP2A down-regulates LPAR5 expression. (A) Q-PCR analysis of LPAR5 transcripts in HONE1 cells or cells expressing EBV-encoded LMP2A or LMP1. (B) Immunocytochemical analysis of LPAR5 in HONE1 cells expressing LMP2A compared to HONE1 vector control cells. NP69 cells were used as a positive control. (C) Reduced expression of LPAR5 by LMP2A was confirmed in two additional NPC cell lines (CNE2 & HK1). * p<0.05; ** p<0.01; *** denotes p<0.001.