# THE ROLE OF LYSOPHOSPHATIDIC ACID IN THE PATHOGENESIS OF ORAL SQUAMOUS CELL CARCINOMA

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**THE ROLE OF LYSOPHOSPHATIDIC ACID IN THE PATHOGENESIS OF ORAL SQUAMOUS CELL CARCINOMA**

## ABSTRACT

Oral squamous cell carcinoma continues (OSCC) is the sixth most common cancer worldwide and accounts for 300,000 new cases yearly with a five-year survival rate of approximately 50%. Current treatment relies on surgery combined with radiotherapy and/or chemotherapy. Major challenges include late presentation, treatment resistance, second primary tumours and other co-morbidities. Thus, there is a compelling need to develop novel therapeutic strategies to improve prognosis and reduce the late burden of the disease and a further understanding into the molecular pathogenesis of the disease could ultimately lead to better management strategies. This study investigated the role of lysophosphatidic acid (LPA) signalling in the pathogenesis of OSCC. LPA is a lipid signalling molecule that regulates many normal physiological processes. LPA is increasingly recognized as being a central mediator of chronic inflammation, which promotes cancer growth, immune invasion, metastasis and treatment resistance. The results of the present study demonstrated that gene expression for the enzyme autotaxin, which produces LPA, and LPA receptor 3 (LPAR3) were upregulated in OSCC tissues. Functionally, LPAR3 was shown to mediate LPA-induced migration and invasion in OSCC cell lines using the LPAR1/3 inhibitor, Ki16425, and shRNA mediated knockdown of *LPAR3*. LPA protected OSCC cells exposed to low doses of radiation via LPAR3, although effect appeared to be cell specific. Possible crosstalk between LPA, EGFR and COX-2 was also investigated. LPA-induced phosphorylation of EGFR was variable, possibly due to high basal levels of phosphorylated EGFR in some OSCC cell lines. By contrast, LPA increased COX-2 levels in all OSCC cell lines examined via LPAR3. Furthermore, LPA-induced OSCC cell migration was attenuated by the COX-2 inhibitor, NS398, and this inhibitor decreased the survival of LPA treated OSCC.

Collectively these data show that COX-2 mediates some of the biological effects of LPA in OSCC cells. Circulating plasma LPA levels in OSCC patients were also investigated for the first time using LC-MSMS. In the present study, the profile of LPA species was different in plasma from OSCC patients compared to normal controls. LPA levels were significantly different among normal, early and advanced groups for LPA 18:0, LPA 18:1 and LPA 20:4. Pairwise comparison between two selected LPA species using scatterplots differentiated the three sample groups (Pair1- LPA 18:1versus LPA 18:2, Pair2 – LPA 18:2 versus LPA 20:4 and Pair3- LPA 18:1 versus LPA 20:4.). Principle component analysis clearly differentiated normal plasma from plasma from both early and advanced cancer stages. Further analysis of the LPA profiles, rather than measuring individual LPA species, might reveal novel pathological or prognostic benefits. In conclusion, LPA synthesis and signalling is deregulated in OSCC. Crosstalk between LPAR3 and COX-2 mediates some of the pro-tumorigenic effects of this lipid which could potentially be exploited to improve the management of this disease. Disruption of this signalling pathway could provide novel therapeutic opportunities for patients with OSCC.

Keywords: LPA, OSCC, migration, radioresistance, crosstalk

**KAJIAN PENYELIDIKAN TERHADAP PERANAN ASID LYSOFOSFATIDIK DI DALAM PATOGENESIS KARSINOMA ORAL SEL SKUAMUS**


**ABSTRAK**

Karsinoma oral sel skuamus (OSCC) adalah kanser keenam paling utama di seluruh dunia. Ia menyumbang sebanyak 300,000 kes baru setiap tahun dengan kadar kelangsungan hidup lima tahun sebanyak 50%. Kaedah rawatan semasa adalah pembedahan yang digabungkan dengan radioterapi dan/atau kemoterapi. Cabaran utama menangani penyakit ini adalah ia hanya dikesan pada peringkat lewat, kerintangan terhadap rawatan, terhasilnya tumor utama kedua dan lain-lain. Oleh itu, terdapat keperluan yang kukuh untuk membangunkan strategi terapeutik terkini untuk memperbaiki prognosis dan mengurangkan beban jangka masa panjang penyakit. Pemahaman lanjut mengenai patogenesis molekul penyakit ini akhirnya boleh menghasilkan strategi pengurusan pesakit dengan lebih baik. Kajian ini menyiasat peranan isyarat asid lysophosphatidik (LPA) dalam patogenesis OSCC. LPA adalah molekul pengisyaratan lipid yang mengawal banyak proses fisiologi normal. LPA semakin dikenali sebagai pengantara pusat peradangan kronik, mempromosikan pertumbuhan kanser, pencerobohan imun, metastasis dan kerintangan terhadap rawatan. Keputusan kajian ini menunjukkan terdapat peningkatan ekspresi gen untuk enzim autotaxin yang menghasilkan LPA dan reseptor LPA 3 (LPAR3) di dalam tisu OSCC. Daripada segi fungsinya, LPAR3 telah bertindak sebagai pengantara migrasi dan pencerobohan yang disebabkan oleh LPA pada sel-sel OSCC dengan penggunaan perencat LPAR1/3, Ki16425, dan dengan mengurangkan ekspresi gen atau ‘knockdown’ menggunakan shRNA untuk *LPAR3*. Apabila didedahkan kepada radiasi pada dos yg rendah, sel-sel OSCC yang dirawat dengan LPA didapati terlindung daripada kesan radiasi tersebut. Walaubagaimanapun, kesan perlindungan ini adalah spesifik kepada sesetengah sel sahaja. Kemungkinan berlakunya interaksi silang di antara LPA, EGFR

dan COX-2 turut dikaji. Kesan fosforilasi teraruh EGFR disebabkan tindakan LPA didapati berbeza diantara sel-sel OSCC. Kesan ini mungkin disebabkan oleh tahap basal fosforilasi EGFR yang tinggi dalam sesetengah sel OSCC. Sebaliknya, LPA didapati dapat meningkatkan ekspresi enzim COX-2 dalam semua sel OSCC melalui perantara LPAR3. Tambahan pula, migrasi sel OSCC yang disebabkan oleh LPA telah direncatkan oleh perencat COX-2, NS398. Perencat ini juga mengurangkan ciri kelangsungan hidup sel-sel OSCC yang dirawat oleh LPA. Secara keseluruhannya, data ini menunjukkan bahawa COX-2 mengantara beberapa kesan biologi LPA dalam sel OSCC. Buat pertama kalinya, paras LPA didalam plasma pesakit OSCC dikaji dengan menggunakan LC-MSMS. Dalam kajian ini, profil spesies LPA didapati berbeza di antara plasma pesakit OSCC berbanding dengan subjek kawalan. Tahap LPA didapati berbeza di antara kumpulan normal, awal dan maju untuk LPA 18:0, LPA 18:1 dan LPA 20:4. Perbandingan pasangan dua spesies LPA yang terpilih dengan plot serakan telah dapat membezakan tiga kumpulan sampel (Pasangan LPA 18:1 dengan LPA 18:2, LPA 18:2 dengan LPA 20:4 dan LPA 18:1 dengan LPA 20:4). Analisis komponen utama jelas dapat membezakan antara kumpulan normal dengan kedua-dua kumpulan tahap kanser awal dan maju. Analisis lanjut mengenai profil LPA secara keseluruhan dan bukannya analisis spesifik spesies LPA, mungkin dapat menghasilkan manfaat patologi atau prognostik baru. Sebagai kesimpulan, terdapat deregulasi sintesis dan isyarat LPA di dalam OSCC. Interaksi silang di antara LPAR3 dan COX-2 mengantarakan beberapa kesan pro-tumorigenik lipid yang berpotensi untuk dieksploitasi dalam memperbaiki pengurusan penyakit ini. Gangguan laluan isyarat ini boleh memberikan peluang terapeutik baru untuk pesakit dengan OSCC.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| AKT | : | Alpha serine/threonine protein kinase |
| ATX | : | Autotaxin |
| BSA | : | Bovine serum albumin |
| cAMP | : | Cyclic adenosine monophosphate |
| C/EBP | : | CCAAT-enhancer-binding protein |
| COX-2 | : | Cyclooxygenase--2 |
| CRC | : | Colorectal cancer |
| DMEM | : | Dulbecco's Modified Eagle Medium |
| DMSO | : | Dimetyl sulphoside |
| cDNA | : | Complimentary DNA |
| EDTA | : | Ethylenediaminetetraacetic acid |
| EGF | : | Epidermal growth factor |
| EGFR | : | Epidermal growth factor receptor |
| *E.coli* | : | *Escherichia coli* |
| ESI | : | Electrospray ionisation |
| FAFA | : | Fatty acid-free human albumin |
| FBS | : | Foetal bovine serum |
| FTY720-P | : | Phosphorylated FTY720 |
| GAPDH | : | Glyceraldehyde-3-phosphate dehydrogenase |
| GCMS | : | Gas chromatography mass spectrometry |
| Gy | : | Gray |
| GPCR | : | G-protein coupled receptor |
| HNSCC | : | Head and neck squamous cellcarcinoma |
| HPV | : | Human papilloma virus |

| | | |
|---|---|---|
| HRP | : | Horse-radish peroxides |
| huR | : | human antigen R |
| IS | : | Internal standard |
| JNK | : | Jun N-terminal kinase |
| LCAT | : | Lecithin-cholesterol acyltransferase |
| LCMS/MS | : | Liquid Chromatography Mass Spectrometry |
| LOH | : | Loss of heterozygosity |
| LNM | : | Lymph node metastasis |
| LPA | : | Lysophosphatidic acid |
| LPAR | : | Lysophosphatidic acid receptor |
| LPC | : | Lysophosphatidylcholine |
| LPL | : | Lysophospholipid |
| LPP | : | Lipid phosphate phosphohydrolase |
| MAPK | : | Mitogen-activated protein kinase |
| β-ME | : | β-mercaptoethanol |
| MMP | : | Matrix metaloproteinase |
| MRM | : | Multiple reaction monitoring |
| mTOR | : | mammalian target of rapamysin |
| MTT | : | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NF-$_{K}$B | : | Nuclear factor-$_{K}$B |
| NPC | : | Nasopharyngeal cancer |
| NPP | : | Nucleotide pyrophosphatase/phosphodiesterase |
| NSAID | : | Nonsteroidal anti-inflammatory drugs |
| NSCLC | : | Non-small cell lung cancer |
| OSCC | : | Oral squamous cell carcinoma |
| PA | : | Phosphatidic acid |

| | | |
|---|---|---|
| PAGE | : | Polyacrylamide gel electrophoresis |
| PBS | : | Phosphate buffered saline |
| PCA | : | Principle component analysis |
| PI3K | : | Phosphoinositide 3-kinase |
| PLC | : | Phospholipase C |
| PLD | : | Phospholipase D |
| PKC | : | Protein kinase C |
| PGE-2 | : | Prostaglandin E2 |
| PTEN | : | Phosphatase and tensin homolog |
| PVDF | : | Polyvinylidene diflouoride |
| PPARδ | : | Peroxisome proliferator-activator receptor-δ |
| QC | : | Quality control |
| qRT-PCR | : | Quantitative Real-Time Polymerase Chain Reaction |
| Rb | : | Retinoblastoma |
| RNA | : | Ribonucleic acid |
| ROCK | : | Rho-associated protein kinase |
| RTK | : | Receptor tyrosine kinases |
| SDS | : | Sodium dodecyl sulphate |
| shRNA | : | short hairpin RNA |
| S1P | : | Sphingosine-1 phosphate |
| SK1 | : | Sphingosine kinase 1 |
| STAT | : | Signal transducer and activator of *transcription* |
| STNMP | : | Site, tumour, node, metastasis, pathology |
| TB | : | Terrific broth |
| TBST | : | TRIS-buffered saline with 0.1% Tween 20 |
| TCGA | : | The Cancer Genome Atlas |

| | | |
|---|---|---|
| TGF-α | : | Transforming growth factor alpha |
| TGF-β | : | Transforming growth factor beta |
| TLC | : | Thin layer chromatography |

# CHAPTER 1:

# INTRODUCTION

## 1.1. General Introduction

Oral squamous cell carcinoma continues to be a serious and growing problem throughout the world. Globally, it accounts for approximately 300,000 new cases each year, with the highest incidence for both males and females seen in Melanesia (Fiji, Papua New Guinea, Solomon Islands and Vanuatu)(Ferlay et al., 2015). The disease is also prevalent in India and South East Asia. The five-year survival rate remains at approximately 50% despite advances in cancer treatment (Warnakulasuriya, 2009). Multiple treatment approaches including surgery, radiotherapy and chemotherapy are used to manage the disease, however major problems such as loco-regional recurrences, the appearance of second primary tumours and distant metastases accounts for poor patient prognosis (Warnakulasuriya, 2009). More improved, strategic and targeted treatment approaches are required to treat this complex disease.

Lysophosphatidic acid is a lipid signalling molecule with a wide variety of biological actions particularly in regulating cell proliferation, migration and survival (Moolenaar, 2000). In serum or plasma, LPA is converted from lysophospholipids by phospholipaseA1/2 and autotaxin whereas in platelets and some cancer cells, it is synthesised from phosphatidic acid by phospholipase D with phospholipase A1/2 (Aoki, 2004). Deregulated LPA signalling has been implicated in the progression of cancer both *in vitro* and *in vivo* and also in the development of resistance to radiotherapy (Brindley et al., 2013).

LPA binds to six known LPA receptors, LPAR1-6. These receptors are coupled with and activate one or more G proteins ($G_{\alpha i/0}$, $G_{\alpha q/11}$, Gs and $G_{\alpha 12/13}$). A number of signalling pathways are activated following the binding of LPA (Yung et al., 2014). The LPA signalling axis has been implicated in cancer. Increased levels of LPA have been reported in ovarian cancer patients (Xu et al., 1998; Yu et al., 2008) and aberrant or elevated expression of the LPARs is found in several human malignancies. Microarray gene expression results of 1700 malignant tissues as compared to 350 normal tissues showed that, LPAR2, 3, 5 and 6 are highly overexpressed in acute lymphoblastic and myeloid leukaemia, but the expression LPAR1 and ATX is relatively low (Willier et al., 2013). Heterogeneous expression of the receptors also contributed to the cellular response of the specific cancer types.

Even though our current understanding on the molecular pathogenesis of oral cancer is expanding novel therapies are still lacking. The first successful targeted treatment using epidermal growth factor receptor antibodies have indicated that with an improved understanding of the molecular events underlying this disease new successful treatment approaches will be developed (Leemans et al., 2011).

### 1.2. General Aims

The survival of OSCC patients has not been markedly improved because problems such as late presentation, locoregional recurrences, development of second primary tumours, resistance to radio-and chemotherapy and distant metastases. Thus, there is undeniable necessity to develop novel therapeutic strategies which could not only improve cure rates, but also reduce the late burden of disease.

Unpublished data from our laboratory showed that treatment of oral cancer cells with LPA *in vitro* stimulates cell motility and stimulates an invasive phenotype in non-malignant HaCaT cells. Therefore, this study was developed to evaluate the therapeutic potential of the LPA signalling pathways in more detail by examining the effects of LPA on the migration, invasiveness and survival of oral cancer cells.

Firstly, the gene expression of LPARs and one of the enzymes involved in its synthesis, ATX, was analysed in OSCC cell lines and tissue samples. As LPA is involved in cell motility, the effects of LPA mediating cell migration and invasion in OSCC cell lines were investigated and the roles of the specific LPARs in these processes examined.

There have been a limited number of studies reporting crosstalk of LPA with epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) pathways. In this study, the ability of LPA to transactivate the EGFR was analysed and potential interactions between LPA and COX-2 investigated. Studies were undertaken to examine whether LPA-induced COX-2 protein expression and the role of COX-2 in mediating LPA-induced cell migration and resistance to radiation were also investigated. This part of the study highlighted two inhibitors that have the potential to be adjunct therapeutic agents in the current existing treatments.

The final section of this study investigated the levels of LPA in plasma of OSCC patients. The profiles of five major LPA species were compared in plasma samples from OSCC patients and normal controls.

## 1.3. Objectives

The objectives of the present study were:

1. To determine the expression of LPARs and ATX in OSCC cell lines and tissues and investigate how LPA/LPAR signalling influences motility and invasion *in vitro*.
2. To investigate the mechanisms and functional significance of cross-talk between LPA, EGFR and COX-2 in OSCC cells.
3. To examine the potential of LPA plasma levels as a biomarker in OSCC.

# CHAPTER 2:

# LITERATURE REVIEW

## 2.1. ORAL CANCER

Oral cancers are malignant neoplasms that arise on the lip, tongue, salivary glands, gingiva, floor of mouth, buccal surfaces and other intra-oral locations according to the International Classification of Diseases (ICD version 9). Squamous cell carcinoma (SCC) of oral mucosal origin accounts for more than 90% of cases (Neville et al., 2002). It is a subgroup of the head and neck squamous cell carcinoma (HNSCC). OSCC can arise from pre-existing potentially malignant disorders such as oral leukoplakia, erythroplakia, submucous fibrosis and lichenoid dysplastic lesions or it can arise *de novo* (Scully et al., 2009)((van der Waal, 2009).

### 2.1.1. Epidemiology of Oral Cancer

Oral cancers accounted for 300,000 cases in 2012 which made up 2.1% of the total cancer cases worldwide. The highest incidence for both male and female was from Melanesia (Fiji, Papua New Guinea, Solomon Islands and Vanuatu) with 22.9 per 100,000 and 16.0 per 100,000 respectively. The rates for men were also comparatively high in South Central Asia (9.9) and in Central and Eastern Europe (9.1). Globally, 145,000 cancer deaths was attributed to oral cancer constituting 1.8% of all cancer death. More than two thirds occurred in less developed regions (Ferlay et al., 2015). In the United States of America, oral cancer makes up 3% of all the tumour cases in men and 2% in women (Neville et al., 2002). According to the Malaysian National Cancer Registry (NCR) 2007, oral cancer is the 21$^{st}$ most common cancer reported in the general population with 353 cases of oral (lip, tongue and mouth) cancer. These cases comprised of 171 males and 182 females. Of all the reported cases with staging, only 35.4% of the

cases were diagnosed at stage I and II. Oral cancer was most prominent among the Indians followed by the indigenous people of Sabah and Sarawak (Zain et al., 1997).

Various potential risk factors have been identified to initiate the process of carcinogenesis in the oral cavity. The most significant risk factors associated with this process are excessive consumption or usage of tobacco, alcohol and betel quid. In a large case control study of oral and pharyngeal cancer with 1114 patients and 1268 controls, tobacco smoking and alcohol drinking have been reported to increase the risks of getting cancer either separately or synergistically (Blot et al., 1988). More than 80% of oral cancer cases were attributed to the usage of tobacco and alcohol (Warnakulasuriya 2009).

### 2.1.2. Clinical Management and Patient Survival

OSCC is characterized by histological appearance and clinical manifestations. Oral carcinogenesis may be associated with precancerous lesions such as leukoplakia (white patch), erythroplakia (red patch) or erythroleukoplakia (mixed red and white lesions) which passes through various phases until the ultimate formation of a cancer. However, the lesions do not necessarily develop into malignant neoplasms (Neville et al., 2002) and clinical studies reported the risk of malignant transformation of oral leukoplakia is in the range of 3.6 to 17.5% (Brouns et al., 2014; Lind, 1987; Silverman et al., 1984). According to their histological appearance, lesions in the epithelium may be classified according to their reactive epithelial changes (hyperkeratosis, hyperplasia and acanthosis) or preneoplastic changes (mild, moderate and severe dysplasia) prior to formation of an invasive carcinoma. The initial classification of lesions is based on the degree of tumour differentiation (well-, moderate- and undifferentiated)(Pindborg et al., 1997). Staging system for oral cancer requires an assessment of the primary tumour (T), lymph nodes

(N) and distant metastasis (M). This staging system is important for establishing proper treatment and determining prognosis (Neville et al., 2002).

Treatment of patients with oral cancer is complex. Various factors are taken into consideration such as location, stage, pathological investigation for surgery and chemotherapy consideration, and identification of targets, dose and fractionation for radiotherapy (Pfister et al., 2011). It is suggested that early stages of the disease (stage I and II) is treated with either surgery or radiotherapy while in the advanced stages, both local and distal is more often treated with multiple types of treatments (surgery, radiotheraphy and chemotheraphy) (Pfister et al., 2011). Surgery is most preferred for resectable oral cavity tumours because of good functional outcome. Radiotherapy is offered to patients who have refused surgery or who have inoperable tumours (Pfister et al., 2011). Despite advances of our knowledge of its epidemiology and pathogenesis, the survival rates of oral cancer have shown little improvement over the past forty years. There is a high risk of developing a second primary cancer and this causes the survival rate continues to be low. The five-year survival rate for oral cancer is around 50%. Cancers of the lips have the best outcome with 90% of the patients surviving for five years whereas hypopharyngeal tumours have the lowest survival outcome. Generally, prognosis decreases with advanced diseases and increasing inaccessibility of the tumour (Warnakulasuriya, 2009). Information on the molecular carcinogenesis of OSCC and the genetic and biological heterogeneity of the disease is scarcely available and this has hindered the progress in designing and implementing new therapeutic strategies. Therefore, a deeper understanding of OSCC pathogenesis is needed to promote the development of improved therapeutic approaches.

### 2.1.3. Molecular pathogenesis of OSCC

Initiation and progression of HNSCC, including OSCC is caused by numerous molecular events that are influenced by intrinsic factors, such genetic predisposition, and extrinsic factors, such as exposure to carcinogens (Califano 1996). The accumulation of genetic changes can lead to formation of premalignant lesions and subsequently to invasive carcinoma. These changes include activation of oncogenes by mutations, amplification or over-expression and inactivation of tumour suppressor genes. The most common genetic alteration in HNSCC is loss of chromosomal region 9p21 in 20% in benign squamous hyperplastic lesions. This region encodes for tumour suppressors p16 and $p14^{ARF}$ genes. The loci for gene alteration was followed by 3p21 (16%) and 17p13 (11%). Chromosome 3p is a region which consists of tumour suppressor genes *FHIT* (fragile histidine triad gene) (Mao et al., 1996). In the next step of the progression which is the dysplasia stage, additional loss of heterozygosity (LOH) occurred at 11q13 (29%), 13q21 (32%) and 14q31 (23%). These losses suggests an early loss event in oral carcinogenesis. Moreover, there is a significant rise in the frequency of LOH from carcinoma *in situ* (CIS) to invasive stage. The loci are 6p (19% and 38%, respectively), 8p (21% and 40%), 8q (20% and 38%) and 4q26-28 (21% and 47%). Furthermore, there is also LOH at 17p13 which is thought to result in the loss of a second *TP53* allele after the inactivation of the first allele by mutation (Califano 1996). Overexpression of cyclin D1 and amplification of 11q13 have been reported in 40% of oral squamous dysplasia (Rousseau et al., 2001). Therefore, loss of chromosome region at 3p, 9p, 17p, 11q, 13q and 14q detected in hyperplasia and dysplasia might represent early indicators of oral carcinogenesis, whereas late stages of carcinogenesis in carcinomas are associated with the losses at 6p, 8q and 4q.

The acquisition of immortality at the dysplasia stage of OSCC progression has been reported and was associated with four changes, namely, loss of retinoic acid receptor (RAR)-β and $p16^{INK4A}$ expression, *TP53* mutation and activation of telomerase. However, one atypical cell line (D17) showed loss of RAR-β and $p16^{INK4A}$ expression but retained functional wild type p53 and telomerase was not activated (McGregor et al., 2002). When ectopic activation of telomerase by transduction of retroviral hTERT expression vector was conducted in D17, telomere was lengthened and D17 became immortalized without any loss of wild-type p53 activity (Muntoni et al., 2003). Increased genomic instability in oral dysplasia is linked to mutation or deletion of *TP53* which may speed up the rate of genetic changes in oral carcinogenesis (Choi et al., 2008). In another study, 18 out of 34 pre-cancer cell cultures showed cancer–associated genetic changes. These changes include somatic mutations (*TP53, NSD1, NOTCH1 and FAT1*) and increasing numbers of chromosomal copy number aberrations (CNAs) (de Boer et al., 2019). Loss of locus at chromosome 9p which consists of the gene *CDKN2A*/ $p16^{INK4A}$ and mutations in *TP53*/p53 were the most prominent, whilst mutations of *NOTCH1* were detected in all cultures originating from surgical margins. This indicates that mutations of *NOTCH1* could be an early occurrence in the development of HNSCC (de Boer et al., 2019).

A comprehensive genomic analysis of 279 HNSCC samples which included both $HPV^+$ and $HPV^-$ tumours were published by The Cancer Genome Atlas (TCGA) consortium. Some examples of the findings includes mutations of *PIK3CA*, novel alterations relating to loss of TNF receptor-associated factor 3 (*TRAF3*) and amplification of *E2F1* in $HPV^+$ oropharyngeal cancers showed aberrant activation of NF-κB, other oncogenic pathways and cell cycle. In $HPV^-$ tumours, there are novel co-amplifications of 11q13 (*CCND1, FADD* and *CTTN*), novel focal deletion of nuclear set domain gene (*NSD1*) and tumour suppressor genes (*FAT1, NOTCH1, SMAD4* and *CDKN2A*).

Recurrent focal amplification of receptor tyrosine kinases (EGFR, ERBB2 and FGFR1) is also predominant in HPV–ve tumours (The Cancer Genome Atlas Network 2015). Moreover, a recent systematic review has identified some of the associated loci of oral cancer. The loci for p53 (17p13.1), EGFR (7p11.2), COX-2 (1q31.1) and PIK3CA (3q26.32) are some of the validated loci associated with oral cancer (Sharma et al., 2017).

Recently, an updated classification of HNSCC has been proposed. A number of cancer genes are involved in the progression of HPV-ve tumours, including CNA-high tumours including *FAT1* and *NOTCH1* with smoking is a known risk factor. There are at least three subgroups for these cancers i.e. basal, classical and mesenchymal (Leemans et al., 2018). The classical subgroup is characterised by mutations of nuclear factor erythroid 2-related factor 2 (*NFE2L2*) pathway. Interestingly, an additional subgroup of HPV-ve tumours has been reported to have wild-type p53, very few copy number aberrations (CNAs), activating HRAS and inactivating caspase 8 (*CASP8*) mutations and to have a more favourable prognosis. Ageing has been hypothesized to be a risk factor for this group. (Cancer Genome Atlas Research et al., 2015) )(Figure 2.1).

HPV has long been considered as a risk factor for HNSCC and it is now known that the vast majority of HPV +ve tumours arise in the oropharynx and HPV is rarely detected at other sites (Castellsague et al., 2016). The detection of E6 and E7 mRNA is considered to be the gold standard in establishing the involvement of the virus, but p16 positivity using immunohistochemistry is recognised as a surrogate marker for HPV (Robinson et al., 2010). In the HPV+ve group, tumours are can be divided into two subgroups, which are the HPV-KRT (HPV-keratinocyte differentiation and oxidative reduction process) and HPV-IMU (HPV-immune response and mesenchymal cell differentiation) (Figure 2.1)(Leemans et al., 2018).

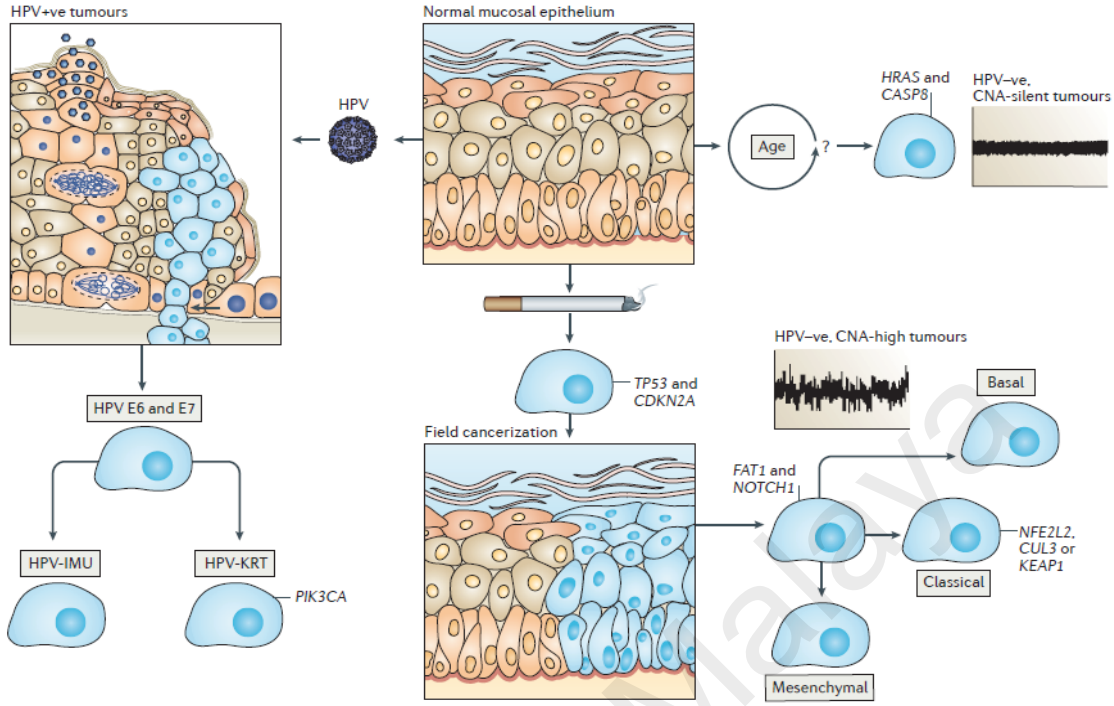



**Figure 2.1: Genomic carcinogenesis model of head and neck squamous cell carcinoma.**

An overview of the current classification of HNSCC. There are four main groups in this classification. The first two groups belongs to tumours with transcriptionally active human papillomavirus (HPV+ve with E6 and E7), HPV-KRT (HPV-keratinocyte differentiation and oxidative reduction process) and HPV-IMU (HPV-immune response and mesenchymal cell differentiation). The second group consists of HPV-ve with wild type TP53 and silent copy number alteration (CNA) tumours and tumours that are HPV-negative (HPV-ve) with high CNA (Leemans et al., 2018).

Recent developments take into account the role of the immune system in the study of HNSCC. HNSCC is an immunosuppressive disease (Ferris, 2015). Several mechanisms have been identified where the tumours can evade immune detection. One example is the increased expression of a transmembrane programmed death-ligand1 (PD-L1) in HNSCCs and enhanced PD-1 expression in cytotoxic T lymphocytes (Leemans et al., 2018). PD-1 inhibitors have been developed, including Nivolumab. Nivolumab is a human IgG4 PD-1 inhibitor antibody that interrupts PD-1 mediated signalling

(Harrington et al., 2017). Affective treatments using Nivolumab has been indicated in a number of solid tumours including HNSCC (Borghaei et al., 2015; Ferris et al., 2016; Homet Moreno et al., 2015; Motzer et al., 2015). In HNSCC, patients treated with Nivolumab had a longer overall survival compared to standard therapy however this response only occurred in 10-20% of the patients (Ferris et al., 2016). The prospect of immunotherapy as a treatment seems promising as it is efficacious in a group of patients, but biomarkers to predict efficacy are needed.

## 2.2. LPA SIGNALLING

### 2.2.1. Lysophosphatidic acid Metabolism

Lysophosphatidic acid (LPA) is a small glycerophospholipid molecule with a molecular weight of 430-480 Da. It exists in all eukaryotic tissues at low concentrations. It is reported to be biologically active at $10^{-9}$ - $10^{-6}$ M and found at higher concentrations (2-5µM) in plasma. LPA is a common name for 1-acyl-glycerol-3-phosphate. Other forms, such as 1-alkyl- or 2-acyl-LPA also exist. The acyl chain length and degree of saturation vary considerably and depends on the precursor phospholipid. Some examples of major LPA species found in the human plasma are shown in Figure 2.2 (Choi et al., 2010). LPA functions as an bioactive lipid mediator with a widespread variety of biological actions predominantly inducing cell proliferation, migration and survival (Moolenaar, 1989; Moolenaar, 2000).

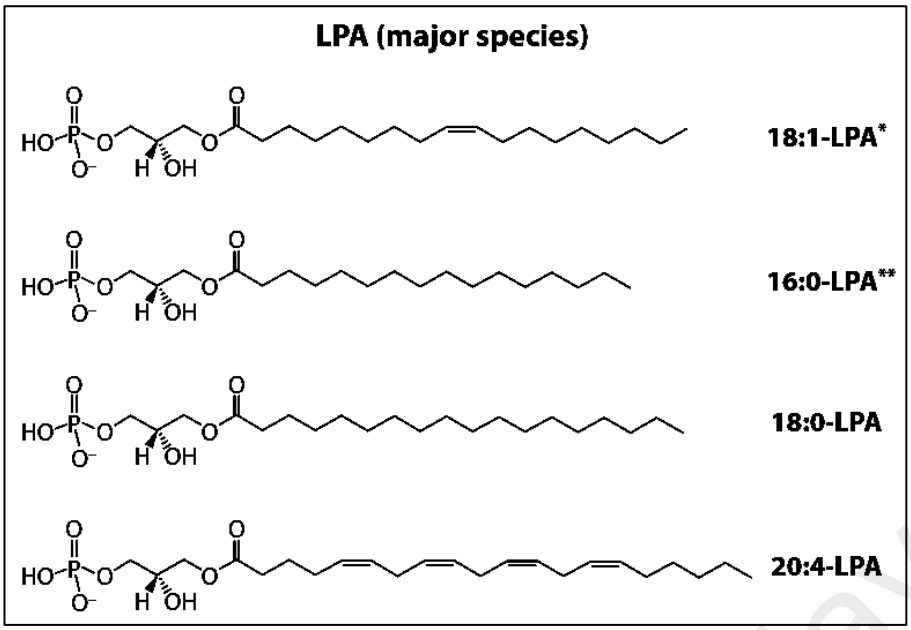



**Figure 2.2: Examples of major LPA species in human plasma.**
18:1 LPA* is frequently utilised in research. The most abundant LPA species in human plasma is 16:0 LPA** (Choi et al., 2010).

### 2.2.1.1. Distribution and Detection of LPA *in vivo.*

Various biological cells and fluids contained substantial amounts of LPA (μM levels). LPA has been found in serum, saliva, seminal fluid, follicular fluid and ascites from ovarian cancer patients. Freshly prepared plasma has a much lower level of LPA compared to serum. This is due to the proposed production of serum LPA as a result of blood coagulation by platelets (Mauco et al., 1978). They have showed that human platelets rapidly produced LPA after treatment with exogenous phospholipase C (PLC) from *Clostridium welchii.* Another study also showed that activated platelets produced LPA but at a very low amount not accounting to the μM level of LPA (Gerrard et al., 1989). LPA has been detected in rat plasma after the plasma was incubated at 37°C for 48 hours (Tokumura et al., 1986). The precursor for LPA synthesis was LPC which is known to exist at high concentrations in plasma (Tokumura et al., 1986). This explained the generation of LPA via the platelet-independent serum pathway. A significant amount of LPA has been detected in saliva and the lipid was able to induce rapid increase in

intracellular free $Ca^{2+}$ concentrations and accelerated cell growth which originated from the mouth, oesophagus and pharynx (Sugiura et al., 2002).

### 2.2.1.2. LPA Production Pathways.

There has been a vast accumulation of knowledge in the physiological roles of LPA through the studies of LPA and its receptors in recent years. Nevertheless, the mechanism of LPA production have not yet been completely elucidated. Described in Figure 2.3 are the hypothetical mechanisms of LPA production (Aoki, 2004). There are two major pathways involved in the synthesis of LPA. There are also other possible pathways involved in the LPA synthesis. LPC is a major precursor for LPA in serum and plasma, whereas, in platelets and some cancer cells, LPA is synthesised from phosphatidic acid. The first pathway is the conversion of phospholipids (PLs) to either 1-acyl-2-lysoPA (LPA) or 2-acyl-1-lysoPA (LPA) by phospholipase $A_1$ ($PLA_1$)/$PLA_2$-LysoPhospholipase D. The second pathway is the conversion of the PLs via PLD-$PLA_1$/$PLA_2$ pathway (Aoki, 2004). Anti-platelet antibodies have been used to deplete platelets in rats to explain the above mechanisms. Analyses of rat serum showed that platelet-dependent pathway generated 50% of serum LPA and the rest was synthesised via a platelet-independent pathway (Aoki et al., 2002).

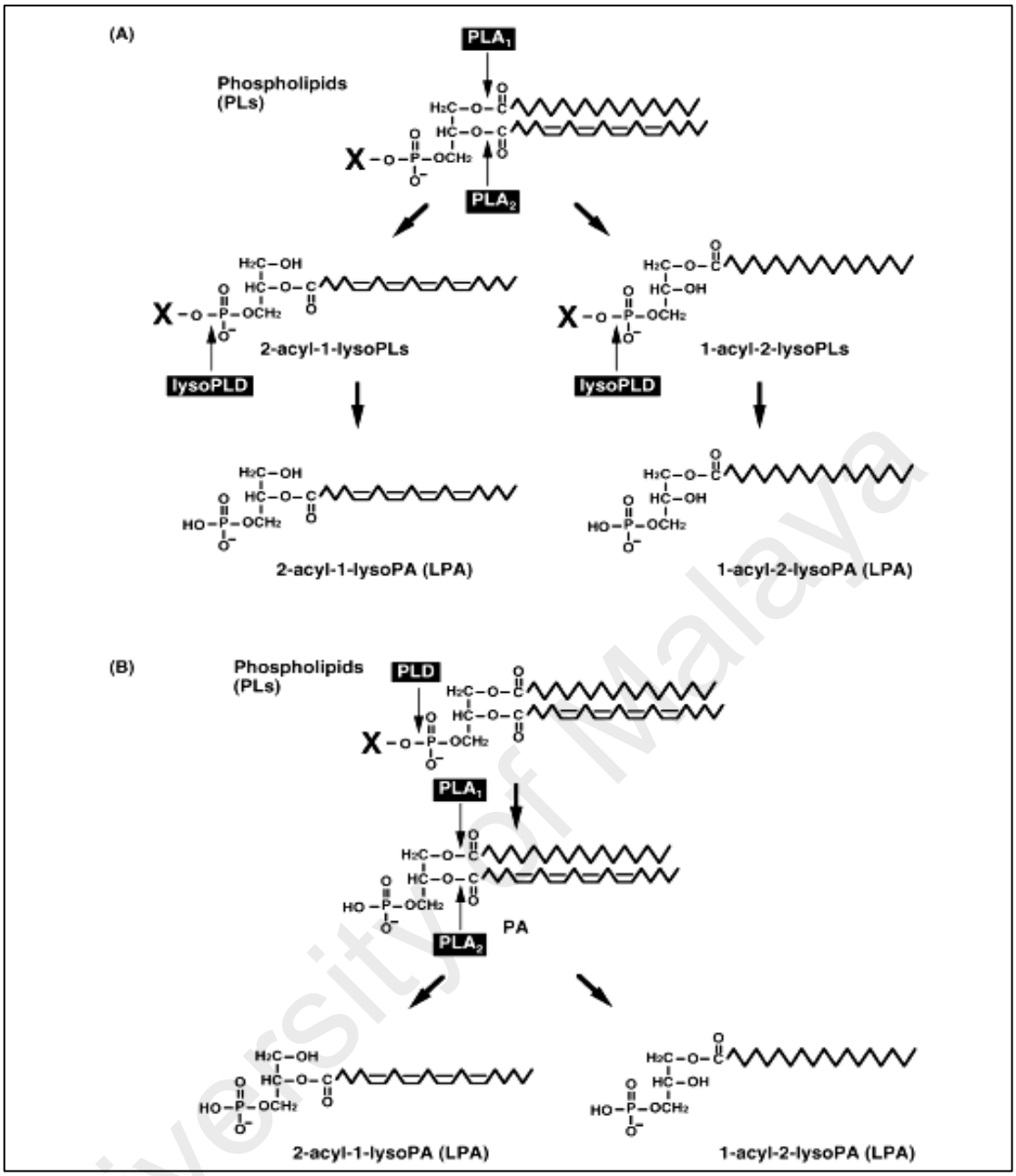



**Figure 2.3: Hypothetical mechanism for the synthesis of LPA**.

(A) PLA1/PLA2-lysoPLD pathway and (B) PLD-PLA1/PLA2 pathway. (Aoki, 2004).

For the platelet-dependent pathway, there are at least three pathways to produce serum LPA (Figure 2.4). The first pathway is possible because a large quantity of LPLs such as Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and

Lysophosphatidylserine (LPS) are converted from the membrane phospholipids of activated platelets by secretory PLA2 (sPLA2-IIA) or PS-PLA1. LPA is formed via the conversion of these LPLs to LPA by lysoPLDs. The second pathway is from the conversion of free PC in the serum to LPC and then to LPA. Two enzymes were involved which are the lecithin-cholesterol acyltransferase (LCAT) and lysoPLD respectively. The third pathway is when activated platelets produce LPA through the hydrolysis of phosphatidic acid (PA) by sPLA2-IIA or membrane-associated PA-selective PLA1 (mPA-PLA1). Even though it is a minor pathway, production of LPA is quick and may play a part in wound healing (Hines et al., 2000).

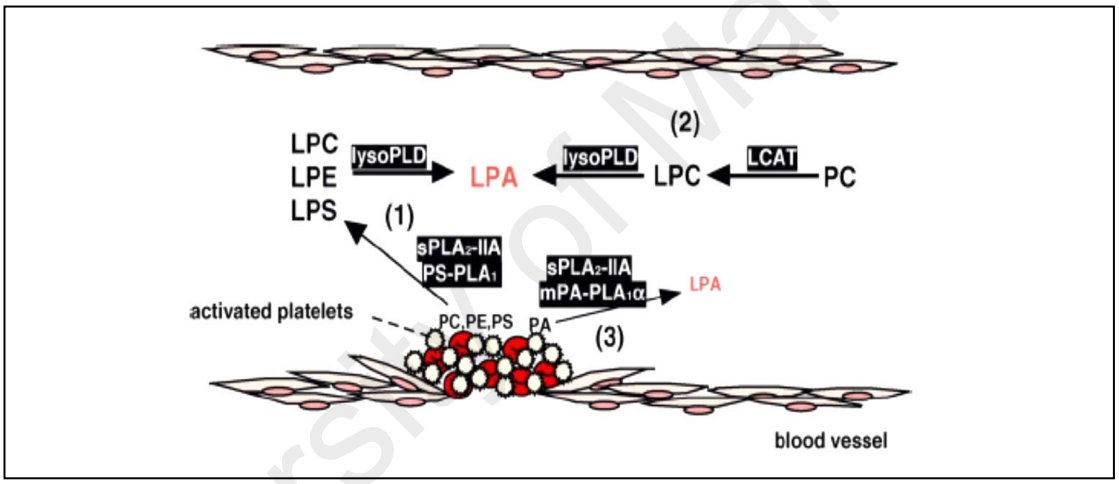



**Figure 2.4: Three pathways for the formation of serum LPA.**
(1) Formation of LPC, LPE and LPS from membrane phospholipids via secretory $PLA_2$ ($sPLA_2$-IIA) or PS-$PLA_1$) followed by LPA synthesis by lysoPLD. (2).Successive synthesis of LPA by the lecithin-cholesterol acyltransferase (LCAT) and lysoPLD. (3) In activated platelets, hydrolysis of PA will produce LPA by sPLA2-IIA or membrane-associated PA-selective PLA1 (mPA-PLA1) (Aoki, 2004).

### 2.2.1.3. LPA Degradation

The formation and action of LPA must be balanced with its inactivation or degradation. Extracellular LPA is degraded by removing the phosphate group generating physiologically inactive monoacylglycerol (van der Bend et al., 1992). The discovery of a family of lipid phosphate phosphohydrolases (LPPs) was later reported where these enzymes facilitate indiscriminate dephosphorylation of LPA and other lipid phosphates

for example phosphatidate (PA) and Sphingosine-1-phosphate (S1P) (Brindley et al., 1998; Sciorra et al., 2002). Mammalian LPPs consists of three isoforms: LPP1, LPP2 and LPP3. These enzymes are integral membrane proteins which are localised on the plasma membrane with the active site on the outer leaflet of the membrane. They are encoded by three separate genes *PPAP2A, PPAP2C* and *PPAP2B* respectively (Tang et al., 2015). The importance of this ecto-activity of LPP1 has been demonstrated in hypomorphic mice where the half-life of circulating LPA increased from 3 minutes to 12 minutes compared to normal controls (Tomsig et al., 2009). Hypomorph is defined as a type of mutation where altered gene products possess a reduced level of activity. It is reported that premature entry into the S-phase of the cell cycle is caused by LPP2 overexpression in fibroblasts, whereas, knockdown of LPP2 delays the entry by approximately 1.5 hours. Over expression or knockdown of LPP1/3 did not show similar results, implying the unique function of LPP2 in cell cycle regulation (Morris et al., 2006). A study with FTY720 demonstrated the roles of LPPs. This molecule is a sphingosine analogue which is utilised as an immunomodulatory drug for treating multiple sclerosis. FTY720 is phosphorylated to FTY720-P by sphingosine kinase-2. Cell lysates of overexpressed LPP1, LPP2 and LPP3 revealed that FTY720-P is only dephosphorylated by LPP3. In whole cells, LPP3 functioned as ectophosphatase regulating the equilibrium between FTY720 and FTY720-P (Mechtcheriakova et al., 2007).

LPPs are also found to be localised in the internal membranes of endoplasmic reticulum (Barila et al., 1996) and Golgi apparatus network (Kai et al., 1997). Intracellular LPPs potentially regulates signal transduction through dephosphorylation of lipid phosphates as opposed to degradation of extracellular LPA or SIP (Tang et al., 2015).

### 2.2.1.4. LPA Receptors.

There are six receptors that have been identified as genuine, high affinity cognate LPAR receptors. The first cell surface receptor for LPA (LPAR1) was identified in 1996 (Hecht et al., 1996). It consists of 364 amino acids with 7 putative transmembrane domains. It is widely expressed in humans especially in the brain, heart, small intestines and etc. LPAR1 couples and activates four types of G proteins, $G_{\alpha i/0}$, $G_{\alpha q/11}$, Gs and $G_{\alpha 12/13}$. Through these G proteins, LPAR1 activation induces the Rho, phospholipase C (PLC), Ras and PI3K pathways (Figure 2.5).

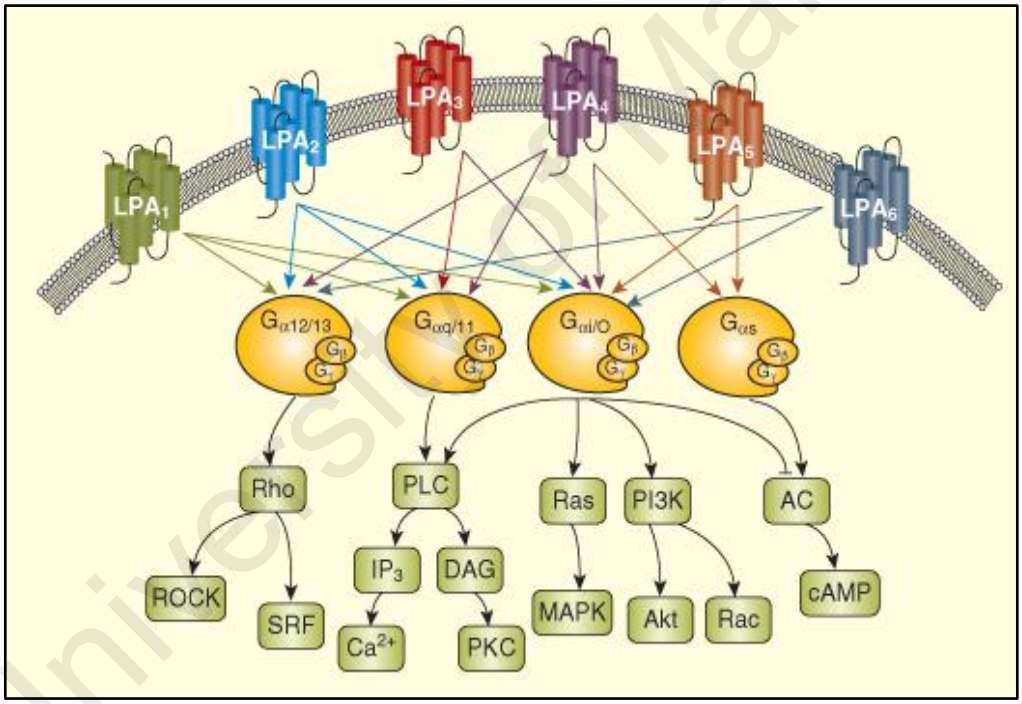



**Figure 2.5: LPA signalling pathways activated by six confirmed LPA receptors** (Yung et al., 2014).

LPAR2 has 60% similarity with LPAR1. LPAR2 encodes a protein that has a 348 amino acid with a calculated mass of about 39kDa. In humans, LPAR2 is highly expressed in testis and leucocytes, and moderate expression in prostate, spleen, thymus and pancreas. LPAR2 couples with $G_{\alpha i/0}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ which is similar to LPAR1. Activation of LPAR2 signalling is associated with cell survival (Deng et al., 2002) and

cell migration (Zheng et al., 2001). LPAR2 has been associated with metastasis in colon (Yun et al., 2005) and ovarian cancers (Yu et al., 2008).

The third receptor, LPAR3 was discovered as another orphan GCPR protein with 50% amino acid similarities to LPAR1. LPAR3 was detected in human heart, testis, prostate, pancreas, lung, ovary and brain. LPAR3 couples with $G_{\alpha i/0}$, $G_{\alpha q/11}$ but not $G_{\alpha 12/13}$ (Ishii et al., 2000). This signalling will later mediate the PLC, Ras and PI3K pathway. LPAR3 has high affinity towards unsaturated fatty acids as opposed to LPAR1 and LPAR2 that prefers saturated fatty acids. (Bandoh et al., 1999; Sonoda et al., 2002).

The fourth receptor, LPAR4 is a receptor with a distinct structure as compared to all other classic LPA receptors found previously. It is more closely related to P2Y purinergic receptors. P2Y purinergic receptor group is one of the three classes of receptors for both ATP and adenosine which is broadly detected in most tissues. One of these classes is a ligand-gated ion channel and the other two are the GPCR receptors. LPAR4 is not activated by any of the nucleotides or nucleosides (Janssens et al., 1997; Noguchi et al., 2003). LPAR4 is consist of 370 amino acids with a calculated molecular mass of around 42kDA. This receptor has specific affinity towards 18:1 LPA (Noguchi et al., 2003). LPAR4 demonstrated independent Rho-mediated neurite retraction and stress fibre formation by coupling with Gα12/13 proteins. It also activated the calcium mobilization via Gαq and increased cAMP mediated by Gαs proteins (Lee et al., 2007).

The fifth receptor is LPAR5 which shares 35% homology with LPAR4 but only 22% homology to LPAR1-3 (Lee et al., 2006). It consists of 372 amino acids with a calculated molecular mass of a 41kDa. LPAR 5 is highly expressed in major tissues of the human body, for example the spleen, the heart, small intestine and placenta. LPA induced neutrite retraction, stress fibre formation and receptor internalization in transfected rat cell lines

expressing LPAR5 by coupling with Gα12/13 and increases intracellular calcium levels via activation of Gαq (Kotarsky et al., 2006; Lee et al., 2006). Binding of LPA to LPAR5 also increases cAMP levels and inositol phosphate production (Kotarsky et al., 2006). Two other lipid-derived molecules (farnesyl pyrophosphate and arachidonylglycine) have been identified as LPAR5 ligands (Oh et al., 2008). Farnesyl pyrophosphate activated Gαq/11 and Gαs-mediated signalling whereas arachidonylglycine acted only through Gαq/11 signalling pathway. Both were suggested to bind to different ligand-binding pocket of LPAR5 (Oh et al., 2008).

LPAR6, previously known as $p2Y_5$ is highly expressed in the epidermis and hair follicles. Several mutations in the *LPAR6* gene lead to autosomal recessive hypotrichosis and a woolly hair type. When *LPAR6* was coexpressed with Gα protein, LPAR6 was activated by LPA and resulted in increased intracellular$CA^{2+}$, reduced forskolin-stimulated cAMP and ERK1/2 activation (Lee et al., 2009)

### 2.2.2. Autotaxin

Extracellularly LPA is synthesised by a secreted lysophospholipase D (LysoPLD) also identified as autotaxin. ATX was first discovered as an autocrine motility factor (125kD) for melanoma cells (Stracke et al., 1992). It was later recognized as a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family and was termed NPP2. ATX undertakes membrane-proximal cleavage producing a soluble exo-enzyme whereas NPP1 and 3 are principally bound to the plasma membrane. The NPP1 and NPP2 can hydrolyse phosphodiester and pyrophosphate bonds in various nucleotides but not ATX (Gijsbers et al., 2003). NPP1 is involved in bone mineralization by generating an inhibitor of calcification called pyrophosphate. NPP3 stimulates glial cells differentiation and invasion of through an unknown mechanism (Goding et al., 2003).

ATX is broadly detected especially in brain, ovary, lung, intestine and kidney. It is also physiologically highly expressed locally in injured tissues to promote wound healing by blood clotting and mobilisation of nearby fibroblasts. (Bourgoin et al., 2010).

### 2.2.3. LPA Signalling

#### 2.2.3.1. Extracellular and Intracellular Actions of LPA

LPA is produced both extracellularly and intracellularly from membrane phospholipids. Intracellular LPA is considered to function as structural component (Coleman et al., 1978) or as an intermediate for phospholipid biosynthesis (Okudaira et al., 2010). Therefore it is less likely to function as a bioactive signalling molecule.

Extracellular LPA acts on six identified LPAR receptors which activate five G-proteins ($G_{\alpha i/0}$, $G_{\alpha q/11}$, $G_{\alpha 12/13}$, $G_{\alpha s}$ and $G_{\alpha 16}$) that are involved in normal cell signalling. These interactions mediate a variety of biological, LPA receptor-mediated functions including mitogenic effects, cell differentiation, cell survival, cytoskeletal reorganization, process retraction and cell migration. These processes involve the function of nervous system, immune system, vascular development, cancer, reproduction, fibrosis and obesity. Table 2.1 summarises some of the expanding range of LPAR signalling effects in normal physiology.

**Table 2.1: Established roles for LPA receptor signalling in normal body systems Adapted from (Choi et al., 2010).**

| HUMAN BODY SYSTEM | PHENOTYPE | ESTABLISHED ROLES FOR LPAR SIGNALING |
|---|---|---|
| Nervous system | Growth or development | 1. Neurogenesis<br>2. Proliferation and differentiation of neural progenitor cells (NPCs)<br>3. Astrocyte proliferation |
| Vascular system | Vasculogenesis<br>Angiogenesis<br>Vasoregulation | 1. Frontal cephalic haemorrhages in LPAR1 & 2 knockout mice.<br>2. Severe vascular defects in ATX null |
| Immune system | T cell functions<br>Dendritic cell functions | 1. Chemotaxis and cytokine production<br>2. Apoptosis |
| Reproductive system | Embryo implantation<br>Spermatogenesis | 1. Timing and spacing of implantation.<br>2. Regulation of prostaglandin pathways<br>3. Sperm motility |

### 2.2.4. LPA Signalling in Cancer

Due to the diverse cellular functions in a variety of body systems, any dysfunction in LPA signalling could contribute to several predominant human diseases for example neuropsychiatric disorders, cardiovascular disease, reproductive disorders and cancer. The connection between binding of LPA to LPAR which activates G proteins and the downstream signalling pathways which result in the induction of transcription factors and cytokine production is shown in Figure 2.6. Ultimately, enhanced cytokine production then contributes to inflammation, hyperplasia, tumour formation and metastasis.

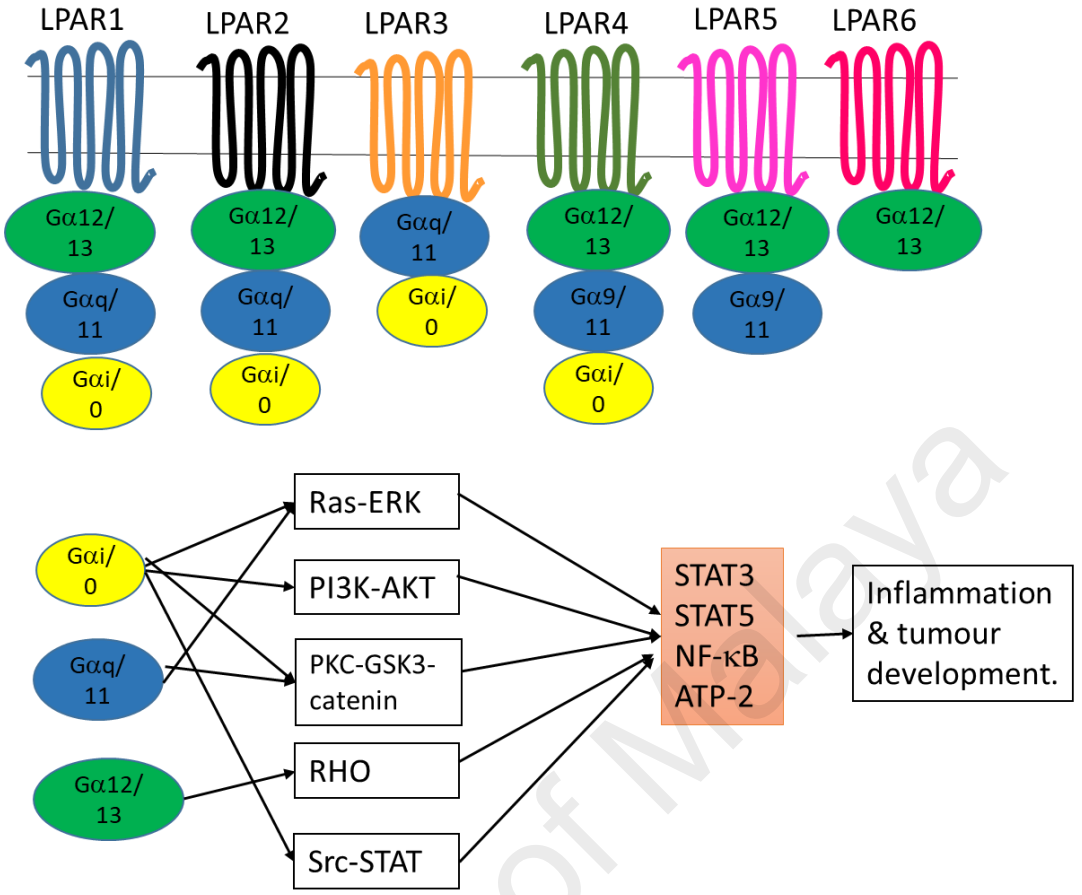



**Figure 2.6: LPA signalling pathways connecting inflammation and cancer.** LPA act on its GPCR via the heterodimeric G proteins Gi, Gq and G12/13 activating Ras-ERK pathway through Gi and Gq, PI3K-AKT pathway through Gi; PKC-GSK3 – catenin pathway through Gq (and/or Gi); Rho CDC42 pathway through G12/13; Src-STAT pathway through Gi all of which induces expression and activation of multiple transcription factors for example STAT3, STAT5, NF-κB, ATF-2 and cytokine production. Enhanced cytokine production contributes to inflammation and cancer. (Liu, Murph, et al., 2009).

Increasing evidence indicates that LPA receptors and ATX play important roles in cancer. Aberrant or elevated expression of LPA receptors are detected in several human malignancies *in vivo* and *in vitro*. Microarray gene expression results of 1700 malignant tissues as compared to 350 normal tissues showed that, *LPAR2*, *3*, *5* and *6* are highly overexpressed in acute lymphoblastic and myeloid leukaemia, but contains relatively little *LPAR1* and ATX expression. Colon carcinoma features high levels of *LPAR3* and *LPAR5* whereas all other receptors and ATX showed relatively low expression. Ovarian cancer displayed high expression of *LPAR3* but low expression for *LPAR5* whereas

stomach carcinomas display the opposite. Interestingly, hepatocellular carcinoma lack overexpression in all LPAR receptors. (Willier et al., 2013). The reasons for diverse expression across tumour entities remain a subject for future studies.

Overexpression of *LPAR2* has been shown to play a role in the pathogenesis of thyroid cancer (Schulte et al., 2001). *LPAR2* and *LPAR3* are significantly overexpressed in the majority of the cell lines of ovarian cancer (Fang et al., 2000) (Fang et al., 2002). A significant overexpression *LPAR6* was detected in squamous cell carcinomas (Gugger et al., 2008). Listed in table 2.2 are some of the established roles of LPA signalling in cancer (Choi et al., 2010).

**Table 2.2: Established roles for LPA signalling in cancer. Adapted from (Choi et al., 2010).**

| | |
|---|---|
| Ovarian | LPA as ovarian cancer activating factor.<br>Potent protumorigenic effect (by LPAR2; partially by LPAR1 and LPAR3).<br>LPAR upregulated.<br>Involvement in hypoxia-stimulated tumorigenic effect. |
| Gastrointestinal | Protumorigenic effect (by LPAR1 and LPAR2).<br>LPAR2-mediated tumour formation.<br>Regulation of known signalling molecules. |
| Lung | Promotion of cancer aggressiveness. |

### 2.2.4.1. Role of LPA in Metastasis, Tumour Invasion and radiotherapy resistance

Metastasis is defined as the spread of cancer cells from the place where they first formed to another part of the body. In metastasis, cancer cells escape from the original (primary) tumour, go through the blood or lymphatic system, and form a new tumour in other tissues or organs in the body (National Cancer Institute dictionary). Genetic instability in the primary tumour drives cell heterogeneity, letting a few chosen metastatic

clones to eventually develop and to spread cancer at a distance (Klein, 2009). Metastasis comprises of successive and discerning steps comprising proliferation, stimulation of angiogenesis, detachment, motility, invasion into bloodstream and crosstalk with components of the new microenvironment (Fidler, 1990; Nguyen et al., 2007). The present study chose to focus on cell motility because it is strictly implicated in invasion and metastasis during the development of cancer (Wells et al., 2013).

Colorectal carcinoma cell lines that showed significant expression of *LPAR1* mRNA responded to LPA by cell migration and production of angiogenic factors (Shida et al., 2003). Overexpression of individual LPARs promoted tumour formation and metastasis in mouse models whereas, knockdowns has the opposite effects. *LPAR1-3* have been found to be sufficient to induce breast cancer in mice, which emphasizes their tumorigenic potential when being expressed in an uncoordinated manner (Liu, Umezu-Goto, et al., 2009). LPAR4 signalling potentially modulates malignant behaviour in HNSCC cells through proliferation and cellular motility (Matayoshi et al., 2013).

Interestingly, LPA has been shown to strongly impede the basal and growth-factor induced migration of B16F10 melanoma cells (Jongsma et al., 2011). This inhibitory effect of LPA is mediated through LPAR5 receptor which increased cAMP and activation of protein kinase A (PKA). These events result in reduced $PIP_3$ signalling which prevents migration of the cancer cells which is possible through the AKT pathway as in Figure 2.6. In another study, *LPAR5* was downregulated in NPC and this down-regulation promoted LPA-induced migration of NPC cell lines (Yap et al., 2015).

ATX/LPA signalling is known to promote radiotherapy resistance (Brindley et al., 2013). One of the proposed hypotheses for this action is when LPA signalling decreases the proapoptotic effect of ceramides (Figure 2.7). Radiation and many chemotherapeutic

agents (Taxol, Doxorubicin, Vincristina etc) cause ceramide to increase in their therapeutic action in inducing cell death. Apoptosis is initiated when ceramide releases cytochrome C from the mitochondria and activate caspases. At the same time, it also blocks the formation of S1P preventing cell survival through a series of reactions involving phospholipase D (PLD) and sphingosine kinase 1 (SK1). The increase of ceramide versus S1P tilts the balance and favours the death over the survival of cancer cells. LPA counteracts these apoptotic effects by increasing the survival signals provided by activating the PLD and PI3K activities. These reactions decreased ceramide formation and increased S1P formation. The ratio of ceramide to S1P is decreased, promoting cell survival relative to cell death (Brindley et al., 2013).

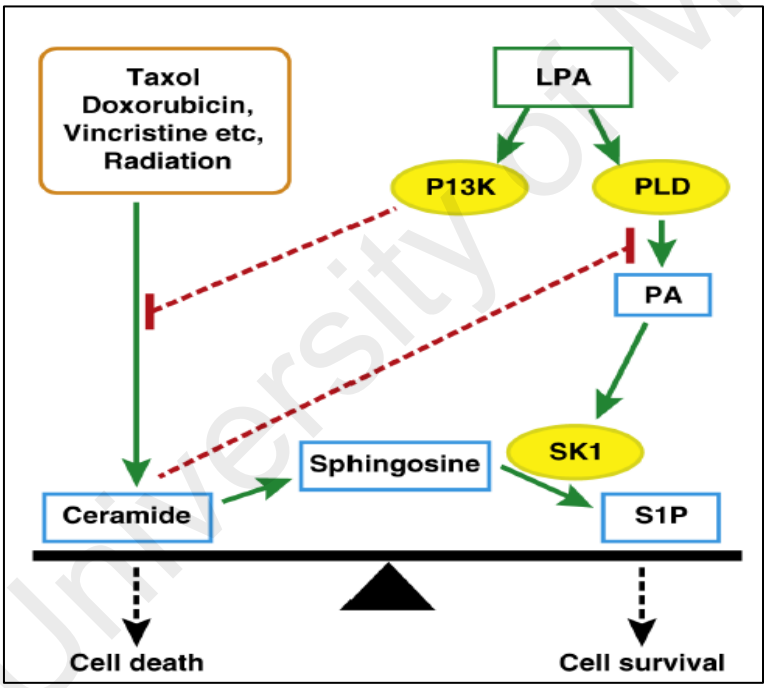



**Figure 2.7: Effect of LPA signalling in radiotherapy resistance.**
This figure showed a proposed hypothesis of the effects of LPA in counteracting the therapeutic effect of radiation and chemotherapeutic agents. LPA activates phospholipase D and PI3K (Brindley et al., 2013).

### 2.2.4.2. Autotaxin and Cancer.

LPC which is the precursor for LPA has been detected in the supernatant of cultured cells. LPC was thought to originate from the shedding of micro vesicles from the plasma membranes (Sciorra et al., 2002). Detachment of micro vesicle has been associated in the metastasis as shown in malignant fluid such as ascites. LPA was also found to be high in ascites which could originates from these high number of micro vesicles in cancer cells. So, ATX is thought to convert micro vesicle-associated LPC into bioactive LPA to explain the connection between detachment of micro vesicle and metastasis.

The discovery of ATX as an autocrine motility factor for melanoma cells itself strengthens the evidence of LPA involvement in initiation and progression of cancer. In these *in vitro* studies, the mRNA level of ATX were found to be overexpressed in several tumour cell types including, neuroblastoma (Kawagoe et al., 1997) hepatocellular carcinoma (Zhang et al., 1999), breast cancer (Yang et al., 2002), renal cell carcinoma (Stassar et al., 2001), glioblastoma (Hoelzinger et al., 2005), non-small cell lung cancer (NSCLC) (Yang et al., 1999), B cell lymphomas (Baumforth et al., 2005) and thyroid carcinoma (Kehlen et al., 2004). *In vivo* studies using nude mice have showed that ATX enhanced tumour aggressiveness (Nam et al., 2000). ATX-transfected breast cancer cells (MCF7) showed increased motility and invasiveness compared to vector-transfected MCF7 cells (Yang et al., 2002). Suppression of ATX in melanoma leads to a 50% reduction of metastasis in a mouse model (Gotoh et al., 2012) which continues to highlight the role of LPA in cellular invasiveness.

ATX has also been linked to tumour invasiveness and aggressiveness. Overexpression of ATX and Ras in transformed NIH 3T3 mouse fibroblasts significantly enhanced metastatic potential compared to Ras transformation alone (Nam et al., 2000). ATX activity in glioblastoma cells accelerates cell motility and adhesion via RAC1 (Ras-

related C3 botulinum toxin substrate and suppression of ATX in melanoma leads to 50% reduction of metastasis in mouse model (Gotoh et al., 2012). ATX levels have been shown to directly correlate with tumour stage and grade in several human malignancies including gastric cancer and non-small-cell lung cancer (Kazama et al., 2011; Yang et al., 1999).

#### 2.2.4.3. LPA Signalling in Oral Squamous Cell Carcinoma

There are only a few studies that have reported the effects of LPA signalling in OSCC specifically or in HNSCC generally. One of the earliest studies reported that treatment of HNSCC cell lines with LPA or a few other GPCR agonists (bradykinin, thrombin, and carbachol) resulted in rapid phosphorylation of the EGFR. This transactivation signal was critically dependent on metalloprotease activity. ERK/mitogen-activated protein kinase pathway was activated in both phosphorylation and tyrosine kinase activities. Critical characteristics of the cell lines such as DNA synthesis, cell cycle progression and tumour cell migration were stimulated by LPA and can be abrogated by inhibiting the EGFR signalling (Gschwind et al., 2002). Another study has shown PI3K pathway played a crucial role on LPA-induced DNA synthesis (cell proliferation) in OSCC cell lines (Aasrum et al., 2016).

In a study on cell migration, LPA was shown to stimulate cell migration on two OSCC cell (E10 and SCC-9) lines but was slightly inhibitory in one cell line (D2). The migration was through LPAR3 and dependent on protein kinase C (PKC) activity (Brusevold et al., 2014). Another receptor, LPAR4 was reported to have the potential to modulate malignant behaviour (cell proliferation and motility) in one of HNSCC cell lines SQ-20B (Matayoshi et al., 2013). Recently, LPAR1 coupled with $G\alpha_i$ has been reported to mediate upregulation of LPA-induced Integrin β6 (*ITGB6*) gene expression. LPA treatment increased phosphorylation and binding of transcription factors SMAD3 and ETS-1 to the *ITGB6* promoter which resulted in *ITGB6* transcription (Xu et al., 2019). Integrin

signalling has been shown to control critical pathways involving cell proliferation, survival and migration (G. J. Thomas et al., 2006). Regulation of integrin beta1 and matrix metalloproteinase-7 by a GTPase activating protein called SIPA1 has been reported to promote migration and invasiveness of OSCC cell lines (Takahara et al., 2017). LPA-mediated overexpression of matrix metalloproteinase-9 regulated by glycogen synthase kinase-3β pathway is crucial in OSCC progression and invasion (Pramanik et al., 2018). In summary, a number of studies have shown the role of LPA in mediating migration and invasion generally in HNSCC and specifically in OSCC.

### 2.3. EGFR Signalling

Epidermal growth factor receptor (EGFR) is a 170kDa transmembrane glycoprotein of a single polypeptide chain. It consist of three domains, extracellular, transmembrane and intracellular. Extracellular domain consist of 622 amino acids with two cysteine rich ligand binding site while the transmembrane domain is a short peptide made of 23 amino acids. The intracellular domain contains 542 amino acids and has a conserved protein tyrosine kinase domain with regulatory tyrosine residue at C-terminal (Carpenter, 1987). EGFR is one of receptor tyrosine kinase groups called the ErbB family. The group contains four ErbB receptors which are ErbB-1 (EGFR), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4).

When the ligands, epidermal growth factor (EGF) or transforming growth factor alpha (TGFα) binds to the extracellular domain, two neighbouring receptors from any of the three known homologs , HER2, HER3 and HER4 will form a dimer and activates subsequent activity of its intracellular tyrosine kinase (Jorissen et al., 2003).

Activated EGFR stimulates many intracellular signalling pathways. Three major pathways activated are Ras-MAPK, PI3K-AKT and PLC. Other important signalling

effectors are signal transducers and activators for example SRC-tyrosine kinase and mammalian target of rapamycin (mTOR), a serine/threonine kinase activated downstream of PI3K-AKT (Hynes et al., 2005; Liebmann, 2011). These multiple signalling cascades regulate multiple cell functions includes cell proliferation, survival, cytoskeletal reorganization and motility (Citri et al., 2006; Mitsudomi et al., 2010). Interestingly, EGF-bound EGFR acts as transcription factor or co-activator of transcription proteins for example as signal transducer and activator to STAT when it translocates to the nucleus (Lin et al., 2001; Lo et al., 2005). Overexpression of EGFR has been reported in many cases of HNSCC (Ozanne et al., 1986). In a clinical trial study, efficacy of the treatment was increased when it consists of radiotherapy together with EGFR-specific antibodies (Cetuximab) while treating patients with HNSCC (Bonner et al., 2006). In a 5-year follow-up study, patients receiving cetuximab-radiotherapy demonstrated a superior overall survival rate than those receiving radiotherapy alone (Bonner et al., 2010). When cetuximab was combined with platinum as the first-line of treatment for recurrent or metastatic HNSCC, the combination therapy resulted in a significantly higher response rate than cisplatin alone (26% versus 10%). However, overall survival was not significantly improved (from 8 to 9.2 months) (Burtness et al., 2005). Another study also showed significantly higher response rate (20% to 36%) and overall survival (from 7.4 to 10.1 months) (Vermorken et al., 2008). Although cetuximab does effect the survival of patients in conjunction with radiotherapy and chemotherapy, many patients are not eligible for both treatments. When cetuximab was given as a monotherapy for platinum-refractory disease, it only demonstrated modest activity (Vermorken et al., 2007). A number of other EGFR inhibitors continue to be tested in HNSCC. These data further demonstrated the essential role of EGFR in the carcinogenesis and its clinical implication in HNSCC.

### 2.3.1. EGFR Signalling in Head and Neck Cancer

EGFR is reported to be overexpressed in 90% of HNSCC. It is associated with local recurrence and poor survival (Ang et al., 2002; Maurizi et al., 1996). EGFR is also reported to be upregulated in histologically normal epithelia adjacent to the head and neck tumour. As the tissue developed to hyperplasia and then to dysplasia, elevated EGFR expression persisted. Moreover, EGFR expression was markedly upregulated during the transition from dysplasia to squamous cell carcinoma, (Shin et al., 1994).

Squamous cell carcinoma consists of around 95% of the cases in head and neck cancers which was previously thought of as a homogenous disease. Insights from RNA and DNA profiling studies have supported that the disease is in fact heterogeneous in nature and cannot be predicted with the current set of clinical markers. Chung et al used gene expression profiling to further divide HNSCC into four subgroups with different prognoses. The group with the worst clinical outcome was the only one to have the activation of EGFR pathway (Chung et al., 2004).

### 2.3.2. Crosstalk between EGFR and LPA Signalling Pathways

A number of studies have described crosstalk between EGFR and LPA signalling pathways. Phosphoinositide 3-kinase (PI3K) was shown to be activated by LPA and required the ability of LPA to transactivate the EGFR signalling pathway (Laffargue et al., 1999). LPA transactivated HER2/neu (ErbB-2) in human gastric cancer cells (Shida et al., 2005b) and LPA-induced effects in colon carcinoma cells was partially dependent on transactivation of EGFR (Mori et al., 2006). LPA-induced EGFR transactivation in human bronchial epithelia cells was regulated by protein kinase C delta, Lyn Kinase and matrix metalloproteinases (Zhao et al., 2006). Another study showed upregulation of sphingosine kinase 1 is mediated by transactivation of LPA with EGFR which promote gastric cancer cell motility and invasion in gastric cancer cells (Shida et al., 2008).

Treatment with LPA was shown to phosphorylate EGFR in three pancreatic carcinoma and two colorectal carcinoma cell lines (Tveteraas et al., 2016). Collectively, these data indicate that some of the pro-tumorigenic effects of LPA are mediated via EGFR in a diverse range of cancer types.

A limited number of studies have reported crosstalk between LPA and EGFR in HNSCC. LPA has been described to cause rapid phosphorylation of tyrosine in EGFR in HNSCC. Disruption of EGFR signalling causes aberrant synthesis of DNA, progression of cell cycle and migration of tumour cells in LPA treated cell lines (Gschwind et al., 2002). LPA-stimulated migration of oral carcinoma cells through LPAR3 acting either in concert with or independent of EGFR transactivation (Brusevold et al., 2014). LPA-induced DNA synthesis in two OSCC cell lines requires transactivation of EGFR through the activation of PI3K pathway (Aasrum et al., 2016).

Currently, binding of LPA as one of the ligands to its GPCR, releases EGF-like ligands tethered at the cell surface via transactivation of EGFR (Figure 2.8) (Lappano et al., 2011). Subsequent intracellular signalling generated will promote cancer progression. Increased understanding of the crosstalk between these two signalling pathway could ultimately facilitate drug discovery and improved prognosis for cancer patients.

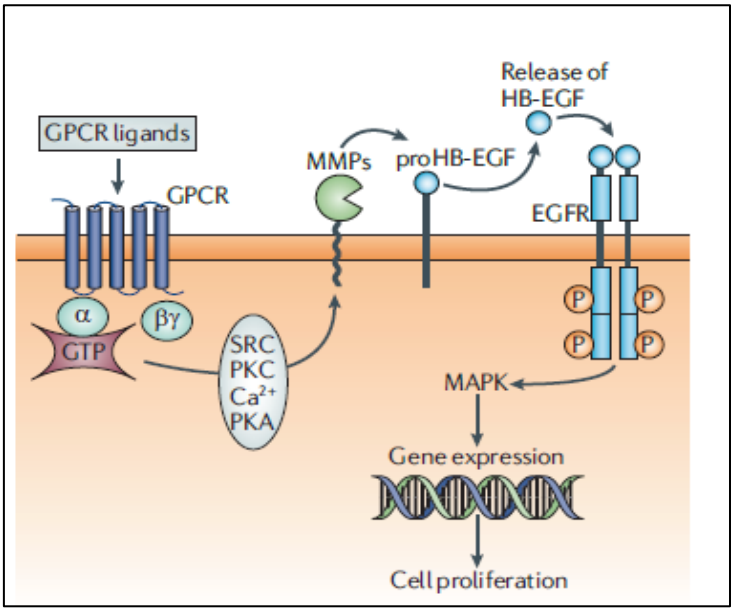



**Figure 2.8: GPCR-induced transactivation of EGFR.**
Binding of ligands to the GPCR stimulates transactivation of EGFR through several intermediaties such as SRC Kinases, $CA^{2+}$, protein kinase C (PKC) and PKA. These processes will activate cleavage of heparin-bound epidermal growth factor (HB-EGF) from proHB-EGF by the matrix metalloproteinase (MMP). Binding of HB-EGF to EGFR activates Mitogen-activated protein kinase pathway. Downstream targets signal induce gene expression and cell proliferation (Lappano et al., 2011).

## 2.4. COX-2 Signalling

Cyclooxygenase (COX) is an enzyme that catalyzes the first two reactions in the prostaglandins (PGs) synthesis. It consists of 581 amino acids with two isoforms COX-1 and COX-2 sharing 60% sequence similarity. A third enzyme COX-3 is found in some specific compartments of the brain and spinal cord (Chandrasekharan et al., 2002; Kis et al., 2003), but the exact function of COX-3 is still under investigation. COX-1 is widely distributed and constitutively expressed in most tissues (platelets, lungs, prostate, gastrointestinal tract, brain, kidney, liver and spleen). Prostanoid products of COX-1 are involved in homeostatic functions. The prostanoids are maintained at the basal rate and increased rapidly when cell membrane remodelling produces increased free arachidonic acids. In contrast, inducible COX-2 is in charge of increasing prostanoid levels in various pathological disorders such as inflammation and cancer and (W. L. Smith et al., 2000).

When the patients suffering from Gardner's syndrome (multiple intestinal polyposis) were treated with NSAIDs, they showed a reduction of adenomas. This observation suggest the role played by COX-2 in tumorigenesis (Waddell et al., 1983). Subsequently, an epidemiological study suggests the link between COX-2 and tumorigenesis where regular intake of aspirin was found to reduce the risk of colorectal cancer (Thun et al., 1991). COX-2 expression was first to be reported to be increased in the colorectal cancer (Eberhart et al., 1994).The role of cyclooxygenases especially COX-2 has been studied in a number of neoplastic diseases, for example in ovarian (Jeong et al., 2008), breast (Glover et al., 2011), gastrointestinal (Cheng et al., 2013), pancreatic (Knab et al., 2014), cervical (Parida et al., 2014) and head and neck cancers (Mendes et al., 2009). Increased COX-2 is shown to decrease the survival rate of some solid tumours (de Groot et al., 2007).

Among all prostanoids, prostaglandin-2 (PGE-2) is the most abundant prostaglandin detected in various human malignancies for example in colon, lung, breast and head and neck cancers, where It is often associated with poor prognosis ((Hambek et al., 2007; McLemore et al., 1988; Rigas et al., 1993; Wang et al., 2004). Prostaglandins bind to cognate cell surface receptors that belong to the G protein coupled receptors (GPCR) family, namely PGE-2 receptor 1-4 (EP1-4) (Wang et al., 2010). Multiple downstream signalling pathways are activated upon the binding of PGE-2 to its receptors to affect numerous processes involved in carcinogenesis (Figure 2.9). PGE-2 stimulates cell proliferation via activation of a glycogen synthase kinase-3β (GSK3β)-β-catenin (β-cat) pathway in colorectal cancer and upregulates aromatase in breast cancer cells. In colorectal cancer (CRC), cell survival is promoted by PGE-2 through the activation of PI3K-Akt-peroxisome proliferator-activator receptor-δ (PPARδ) cascade in both *in vitro* and *in vivo* experiments. CRC cell migration and invasion induced by PGE-2 is activated

by β-arrestin-1-SRC-EGFR-PI3K-Akt signalling *in vitro* and in *vivo*. PGE-2 also induces cell migration and invasion through Erk-ETS-MMP2 cascade in pancreatic cancer cell lines or upregulates C-C chemokine receptor 7 (CCR7) in breast cancer cell lines (Wang et al., 2010).

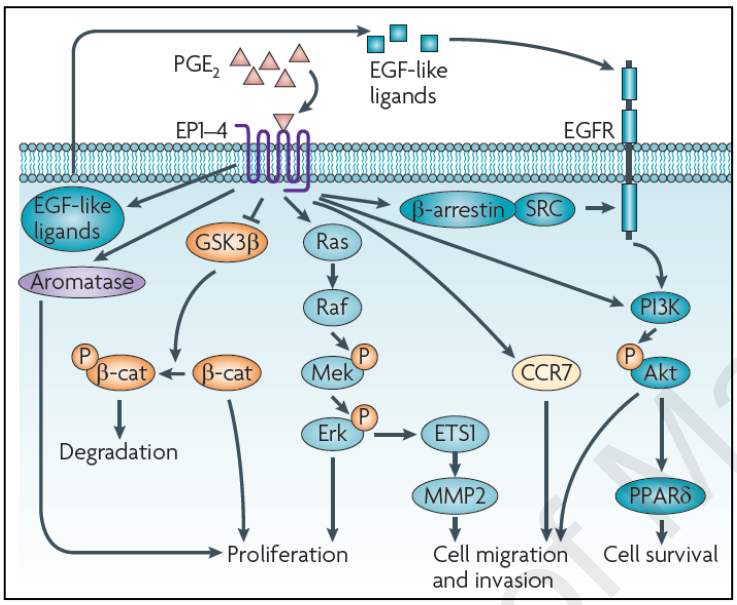



**Figure 2.9: Prostaglandin-2 (PGE-2) promotes cancer progression in tumour epithelial cell proliferation, survival, migration and invasion.**
Downstream pathways for cell proliferation are activated through upregulation of aromatase, GSK3β-β-catenin and Ras-Raf-Mek-Erk pathways. Cell migration and invasion is stimulated via Ras-Raf-Mek-Erk-MMP2, upregulation of CCR7 and β-arrestin-1-SRC-EGFR-PI3K-Akt. Cell survival is stimulated through PI3K-Akt-peroxisome proliferator-activator receptor-δ (PPARδ) cascade (Wang et al., 2010).

### 2.4.1. COX-2 Signalling in Head and Neck Cancer

Several studies have reported the overexpression of COX-2 in HSNCC (Byatnal et al., 2015; Chan et al., 1999; Gallo et al., 2002; Shibata et al., 2005). Expression of COX-2 increases from the lowest in normal epithelium, followed by in hyperplasia, dysplasia and the highest in invasive squamous cell carcinoma. This suggest the involvement of COX-2 in the oral carcinogenesis (Renkonen et al., 2002). A positive correlation between COX-2 expression and severity of dysplasia is shown in OSCC (Seyedmajidi et al., 2014) and

expression at both the mRNA and protein levels are increased in oral epithelial dysplasia as it progressed to OSCC (Nagatsuka et al., 2012).

In OSCC, COX-2 has been suggested to be an important biomarker of prognostic significance that can be used to categorise high risk patients and to estimate patient survival (Byatnal et al., 2015). In a meta-analysis, elevated expression of COX-2 may be associated with pathogenesis of oral cancer and with worse prognosis in oral cancer patients (Wang et al., 2014).

### 2.4.2. Crosstalk between COX-2 and LPA Signalling Pathways

Binding of exogenous LPA to GPCRs results in activation of many signalling pathways such as mitogen-activated protein kinases (MAPK)(Saatian et al., 2006), phospholipase D (PLD)(Wang et al., 2003) and transcriptional factors such as nuclear factor KB (NF-KB)(Cummings et al., 2004), Jun-N-terminal kinase (JNK) (Saatian et al., 2006). LPA was suggested to induce transcriptional regulation of COX-2 expression through EGFR-transactivation dependent activation of transcription factor C/EBPβ and EGFR-transactivation independent activation of NF-KB and JNK in human bronchial epithelial cells (Figure 2.10) (He et al., 2008). In another study with feline oesophageal epithelial cells, LPA-induced COX-2 expression requires activation of PKC and ERK1/2 downstream of LPAR1/3 (Kim et al., 2008).

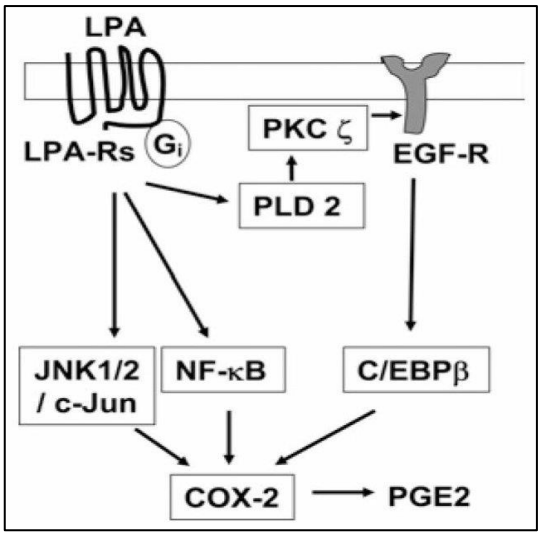



**Figure 2.10: Transactivation of EGFR induced by LPA regulates the expression of COX-2 and releases PGE-2 by activating C/EBPβ in human bronchial epithelial cells** (He et al., 2008).

In ovarian cancer cells, LPA induced robust and sustained expression of COX-2 through transcriptional and post-transcriptional mechanisms, mediated by transcription factor C/EBP and the mRNA stability protein, respectively (Oyesanya et al., 2008). In another study where ovarian cancer cells expressed high levels of LPAR2, LPA is shown to induce expression of COX-2 in dose dependent manner. Pre-treatment of the cells with Gi (pertussis toxin), Src, EGFR and ERK inhibitors reduced LPA-induced expression COX-2 and transfection of cells with LPAR2 siRNA significantly inhibited LPA-induced activation of EGFR and ERK as well as COX-2 expression. These results show the significance of LPAR2 and Gi/Src pathway for LPA-induced COX-2 expression (Jeong et al., 2008). COX-2 was also increased following LPA–mediated transactivation of EGFR via Ras/MAPK pathway in ovarian cancer cell lines (Symowicz et al., 2005) and LPA transactivated both c-Met and GFR which induces COX-2 expression in human colon cancer (Shida et al., 2005a).

There is increasing evidence to show a connection between LPA signalling, EGFR and COX-2 pathway in cancer but no studies have been conducted in head and neck cancer.

## 2.5. Targeting the LPA Pathway for Cancer Therapeutics

Currently, the main component for initial treatment of localised advanced HNSCC is definitive local therapy (surgery and/or radiation therapy (RT)). Unfortunately, this procedure is associated with high rates of loco regional and distant recurrence. In addition, these treatments can also cause considerable morbidity including loss of swallowing and larynx function. In order to improve the cure rates and functional outcomes, chemotherapy has been integrated into the treatments, but disease recurrence still represents a major problem. More precise or targeted treatments would mould likely increase the chance of managing this disease.

The importance of the ATX-LPA signalling has been heightened making it an emerging target for cancer therapy. Strong evidence has implicated LPA in tumorigenesis such as promoting proliferation and angiogenesis. Deregulated LPA signalling via the aberrant expression of the enzymes that generate LPA and the LPARs suggests that this pathway may be a good target for therapeutic intervention. Specific targets include the LPA molecule itself, LPA receptors and autotaxin. Listed below in table 2.3 are the targets and some selective antagonists of the LPA signalling pathway.

**Table 2.3: The targets and some examples of selective antagonist of the LPA signalling pathway. Adapted from (Murph et al., 2007)**.

| Target | Compound | Reference |
|---|---|---|
| LPA | Lpathomab™ | Lpath Inc (http://www.lpath.com) |
| LPAR1 | Ki16425<br>DGPP 8:0<br>PA 8:0<br>Phosphonothioate & fluoromethylene phosphonate<br>VPC-32183<br>VPC-12299 | (Ohta et al., 2003)<br>(Durgam et al., 2006)<br>Fischer, Nusser et al. 2001)<br>(Xu et al., 2006)<br>(Lee et al., 2005)<br>(Heasley et al., 2004) |
| LPAR2 | Farnesyl phosphates | (Liliom et al., 2006) |
| LPAR3 | Ki16425<br>DGPP 8:0<br>PA 8:0<br>Phosphonothioate & fluoromethylene phosphonate<br>VPC-12299 | (Ohta et al., 2003)<br>(Durgam et al., 2006),<br>(Fischer et al., 2001)<br>(Xu et al., 2006)<br>(Heasley et al., 2004) |
| LPAR4 | α-bromomethylene phosphonate | (Jiang et al., 2007) |
| Autotaxin | 2ccPA 16:1, 3ccPA 16:1<br>2ccPA 18:1, 3ccPA 18:1<br>L-histidine<br>GLPG1690™ | (Baker et al., 2006)<br><br>(Clair et al., 2005)<br>(Matralis et al., 2018), (http://www.glpg.com/IPF). |

Recent breakthroughs have been reported in targeting aberrant signalling in cancers involving tyrosine kinase receptors and growth factors using monoclonal antibodies. Some examples are Erbitux® targeting EGFR, Avastin® targeting VEGF and Herceptin® targeting ErbB2/Her2 receptor. LPath Therapeutics (San Diego, CA, USA) has generated a monoclonal antibody that targets all extracellular LPA called

Lpathomab®. It has undergone the first phase of the clinical trial in 2016, in a double blind and placebo controlled study using a single ascending dose to assess the safety, tolerability, immunogenicity and pharmacokinetics of Lpathomab® in healthy volunteers. It was reported to be well tolerated at all doses tested with no serious adverse events or dose limiting toxicities were observed (http://www.lpath.com/).

### 2.5.1. Targeting the Autotaxin in Cancer

ATX has been widely documented to have a role in invasion, aggressiveness and tumour development as discussed previously. There are at least 164 of ATX inhibitors have been reported generated by the academia and industry (Matralis et al., 2018). These inhibitor belongs to three major groups called metal chelators, substrate- and lipid-based inhibitors and small ATX inhibitor molecules with drug-like properties. Subgroups of the third groups are boronic acid-based inhibitors, pipemidic acid-based inhibitors, PF8380-based inhibitors, indole-based inhibitors, imidazopyridine-based inhibitors, benzene sulphonamide-based inhibitors, dual-acting inhibitors, hydroxamic acid-based inhibitors and other types of ATX inhibitors (Matralis et al., 2018). The first ATX inhibitor that have entered advanced clinical trial is GLPG1690. It is a drug treating against idiopathic pulmonary fibrosis (IPF). It has successfully completed phases 1 and 2a clinical trials with some promising results. It is now being evaluated in phase 2b trials (http://www.glpg.com/IPF).

### 2.5.2. Targeting the LPARs in Cancer

A number of inhibitors has been developed to target LPARs. For example, KI16425, DGPP 8:0, PA 8:0, VPC12249 are all LPAR1 and LPAR3 antagonists. Both receptors are able to activate two G proteins and the related downstream signalling pathways therefore having one inhibitor that could target both is advantageous (Liu, Murph, et al., 2009).

Another strategy is to target both ATX and LPAR to control tumour growth and metastasis (Gotoh et al., 2012). There are a few inhibitors that could inhibit both targets. Carba-cyclic phosphatidic acid is an LPA analogue that is able to inhibit ATX without activating any of the LPAR1-4. It has been shown to inhibit MM1 carcinoma and A2058 melanoma metastasis in mice. (Baker et al., 2006; Gupte et al., 2010; Uchiyama et al., 2007). Several lipid analogues for example LPA bromophosphate enantiomers (BrP-LPA) have been shown to inhibit ATX while functioning as LPAR antagonists. The two forms, syn and anti diastereomers of BrP-LPA are pan-LPA GPCR antagonists and a nanomolar inhibitor of ATX. The anti-isomer form was more effective in reducing cell migration and invasion of breast cancer cell lines (Zhang et al., 2009).

### 2.5.3. Ki16425

The discovery of Ki16425 was established by screening 150,000 compounds established on the capability to inhibit $Ca^{2+}$ response to LPA (Ueno, 2001 patent). Ki16425 is an isoxazole derivative found to inhibit LPA-induced action of LPA receptors with high specificity. It also inhibited LPA-induced DNA synthesis and cell migration (Ohta et al., 2003). Several studies have utilized Ki16425 to prove the contribution of LPAR1 and/or LPAR3. For example, treatment with ascites induced pancreatic cancer cell migration and Ki16425 is able to inhibit this observation. However, cell migration continues when the experiments were repeated using a specific LPAR3 inhibitor DGPP 8:0. This result suggested the involvement of LPAR1 instead of LPAR3 in the migration response to ascites in pancreatic cell lines (Yamada et al., 2004). In another study, ascites from pancreatic cancer patients have been reported to stimulate pancreatic cell migration through LPA and LPAR1. This action was inhibited by Ki16425. However, this study

also showed that LPAR2 that are coupled with G(12/13) protein/Rho signalling, was able to inhibit EGF-induced migration and invasion of pancreatic cells (Komachi et al., 2009).

In the HNSCC cell line SQ-20B, LPA-stimulated cell proliferation was attenuated by Ki16425 and Rac1 inhibitor but not Y-27632 (Rho-associated coiled-coil forming kinase (ROCK) inhibitor). This suggest the role of Gi-Rac signalling axis may play a role in downstream signalling of LPAR1/3 activation. Meanwhile, all three inhibitors suppressed LPA-stimulated cell motility in the same cell line suggesting the participation of both G12/13-Rho-ROCK and Gi-Rac signalling pathways via LPAR1/3 activation (Matayoshi et al., 2013).

### 2.5.4. NS398

Epidemiological studies have suggested that chronic intake of aspirin may reduce the risk of developing colon and colorectal cancer (Giovannucci et al., 1995; Thun et al., 1991). Aspirin belongs to an important group of drugs called nonsteroidal anti-inflammatory drugs (NSAIDS). NSAIDs are used to treat inflammatory conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA). These traditional NSAIDS however are associated with gastrointestinal (GI) toxicities associated with widespread usage in long term therapy (Tamblyn 1997). Later, selective COX-2 inhibitors were developed in order to produce better anti-inflammatory and analgesic agents with lessened bad side effects compared to previous NSAIDS.

Selective COX-2 inhibitors have shown to have anti-proliferative and pro-apoptotic effects on a number of cancer cell lines suggesting the use of NSAIDS in the treatment of cancer (Harris et al., 2005; Wang, 2005). Even though the COX-2 enzyme is targeted by for a number of NSAIDS through COX-dependent mechanisms, some NSAIDS also act via COX-independent mechanisms (M. L. Smith et al., 2000).

An example of selective COX-2 inhibitor is called NS398 which is a structural analogue of nimesulide. NS398 is often used in biological experiments as a COX-2 selective inhibitor and anti-proliferative and a pro-apoptotic agent in a number of cancer cell lines (Renard et al., 2006). Some examples of the use of NS398 in research are described. NS398 was shown to suppress proliferation of gastrointestinal cancer cell lines that highly expressed COX-2. However the effect was less pronounced in cell lines with significantly lower levels of COX-2 (Tsuji et al., 1996). The same effect was shown in gastric adenocarcinomas that have overexpression of COX-2 (Sawaoka et al., 1998). NS398 also inhibits OSCC cell proliferation by mechanisms dependent and independent of reduced PGE-2 synthesis (Minter et al., 2003). It was also shown to strongly supress proliferation in OSCC cell lines (Ko et al., 2008). Treatment with NS398 was shown to supressed invasiveness of OSCC cell lines through down-regulation of matrix metalloproteinase-2 and CD44 (Kinugasa et al., 2004). NS398 inhibited specifically alpha (v)beta(6)-dependent integrin in OSCC *in vitro* and *in vivo*. This effect was modulated through PGE-2-dependent activation of Rac-1 (Nystrom et al., 2006). Due to its availability and good efficacy, NS398 was selected for use in this present study.

# CHAPTER 3:

# METHODOLOGY.

## 3.1 Tissue Samples

Samples of five normal oral mucosa and 52 OSCCs biopsies were obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS) managed by Oral Cancer Research and Coordinating Centre (OCRCC) University of Malaya (Zain et al., 2013). Samples were collected either during biopsy, surgery or follow up from patients with consent who had visited University Malaya Medical Centre from 2005 to 2011. The OSCC tissue specimens were derived from the tongue (excluding the base of the tongue), buccal mucosa, gum, palate, floor of the mouth and lip. The tumour tissues were surgical excision specimens and the normal samples were obtained from normal oral mucosa adjacent to impacted wisdom teeth during surgical removal of the impacted teeth. Tissues were snap frozen and kept in liquid nitrogen. RNAs were extracted routinely from the frozen tissue using extraction kits according to the manufacturer's protocol (RNeasy micro kit) (Qiagen, Hilden, Germany). Information regarding the age, gender, risk factors, cancer stages and lymph node metastasis (LNM) are shown in Table 3.1. The American Joint Committee on cancer staging criteria was used for tumour staging (Paleri et al., 2010). Written informed consent was obtained before sample collection and ethical approval for this study was obtained from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DF OB1403/0009L).

**Table 3.1: Clinicopathological characteristics of OSCC and normal individuals.**

| Characteristics | Tumour (n=52)(%) | Normal (n=5)(%) |
|---|---|---|
| Gender | | |
| Male | 18 (34.6) | 3(60) |
| Female | 34 (65.4) | 2(40) |
| Age (years) | | |
| ≤50 | 17 (32.7) | 5 (100) |
| ≥50 | 35 (67.3) | 0 (0) |
| Smoking status | | |
| Smokers | 18 (34.6) | 3 (60) |
| Non-smokers | 34 (65.4) | 2 (40) |
| Betel chewing status | | |
| Chewers | 24 (46.2) | 0 (0) |
| Non-chewers | 28 (53.8) | 5 (100) |
| Stage | | |
| Early (I and II) | 19 (36.5) | N/A |
| Advanced (III and IV) | 33 (63.5) | N/A |
| LNM status | | |
| Positive | 26 (50) | N/A |
| Negative | 26 (50) | N/A |

LNM refers to lymph node metastasis.
N/A- not available.

## 3.2 Cell Lines

### 3.2.1 Human OSCC-derived Cell Lines

Eight human oral squamous cell lines (H103, H157, H314, H357, H376, H400, H413 and BICR31) were used in this study (Edington et al., 1995; Prime et al., 1990). The cell lines have been characterized with regards to *HRAS* (Clark et al., 1993; Yeudall et al., 1993), *TP53* (Burns et al., 1993; Yeudall et al., 1995) and p16 (*CDKN2A* gene) (Loughran et al., 1996; Munro et al., 1999; Wu et al., 1999) mutations and tumorigenicity by orthotopic transplantation to the floor of the mouth in athymic mice (Bulb/C; nu/nu) (Tables 3.2 and 3.3) (Prime et al., 2004). Table 3.4 shows the clinical classification of the original tumours from which the cell lines were derived.

**Table 3.2: Characteristics of the human malignant oral keratinocytes cell lines.**

| Cell lines | *HRAS* mutation codon[1] | *TP53* mutation codon (exon)[2] | *CDKN2A*[3] Mutation | *CDKN2A*[3] Methylation | Modal chromosome number[4] |
|---|---|---|---|---|---|
| **H103** | WT | 244(7) G-T | *p16* R58X (nonsense) | - | 41 |
| **H157** | WT | 306(8) G-A | *p16* R80X (nonsense) | - | 88 |
| **H314** | WT | 176(5) G-T.373 (11) A-G | *p16* 205delG (premature stop codon downstream) | - | 72 |
| **H357** | 13 G-A. 61 A-G | 110(4) G-A | *p16* R58X (nonsense), 3'UTR base 500 C>G | - | 83 |
| **H376** | WT | 266(8) G-T | WT | + | 75 |
| **H400** | WT | 283(8) C-G | WT | + | 82-91 |
| **H413** | WT | 68(4) A-G | WT | + | 76 |
| **BICR31** | WT | 173, 174 36p deletion | Deletion | - | NK |

1. (Clark et al., 1993; Yeudall et al., 1993).

2. (Burns et al., 1993; Yeudall et al., 1993).

3. (Loughran et al., 1996; Munro et al., 1999; Wu et al., 1999).

4. (Patel et al., 1993).

WT.wild type; NK, not known; +, present; -, absent

**Table 3.3: Tumorigenicity of human malignant oral keratinocyte cell lines following orthotopic transplantation to the floor of the mouth in athymic mice.**

| Cell line | Primary tumour | | Characteristics of Dissemination[3] | | | |
|---|---|---|---|---|---|---|
| | Tumour uptake[1] | Differentiation[2] | Local Spread | | Distant Spread | |
| | | | Vascular | Bone | Lymph node | Lung/liver |
| **H 103** | 13/16 | 12 W, 1 M | 2/13 | 0/13 | 0/13 | 0/13 |
| **H 157** | 0/13 | - | - | - | - | - |
| **H 314** | 14/15 | 14 M | 12/14 | 1/14 | 4/14 | 0/14 |
| **H 357** | 0/24 | - | - | - | - | - |
| **H 376** | 0/14 | - | - | - | - | - |
| **H400** | 18/27 | 13 W, 5 M | 0/18 | 0/18 | 0/18 | 0/18 |
| **H413** | 6/9 | 6 M | 0/6 | 0/6 | 0/6 | 1/6[4] |
| **BICR31** | 20/20 | 6W, 7M,7M/W | 14/20 | 6/20 | 3/20 | 0/20 |

1. Results are expressed as the number of animals with primary tumours per number of animals transplanted.
2. WD, well differentiated; MD, moderately differentiated; M/WD, moderately to well differentiated.
3. Results are expressed as the number of cases with either tumour invasion of blood vessels, lymphatics or bone, or with metastases in lymph nodes or lungs, per number of primary tumours.
4. Lung metastasis

(Prime et al., 2004).

**Table 3.4: Clinical characteristics of the origin of human malignant oral keratinocyte cell lines**

| Cell Line[1] | Age | Sex | Site[1] (S) | Size (mm) (T) | Nodal spread[2] (N) | Metastases[3] (M) | Pathology[4] (P) | STNMP Grade[5] |
|---|---|---|---|---|---|---|---|---|
| **H 103** | **32** | **M** | **T** | **<20** | **-** | **-** | **W** | **I** |
| **H 157** | **84** | **M** | **BM** | **20-40** | **+** | **-** | **W** | **II** |
| **H 314** | **82** | **M** | **FOM** | **20-40** | **+** | **-** | **M** | **II** |
| **H 357** | **74** | **M** | **T** | **<20** | **-** | **-** | **W** | **I** |
| **H 376** | **40** | **F** | **FOM** | **20-40** | **+** | **-** | **W** | **III** |
| **H400** | **55** | **F** | **AP** | **20-40** | **-** | **-** | **M** | **II** |
| **H413** | **53** | **F** | **BM** | **20-40** | **-** | **-** | **M** | **II** |
| **BICR31** | **NK** | **NK** | **T** | **<20** | **-** | **-** | **M** | **IV** |

1. Tongue (T), buccal mucosa (BM), floor of mouth (FOM), alveolar process (AP), hypopharynx (H).
2. +, lymph node spread; -, without lymph node spread.
3. +, with metastatic spread; -, without metastatic spread.
4. W, well differentiated; M, moderately differentiated.
5. STNMP grading is a prognostic indicator for oral squamous cell carcinoma with 51.5%, 40.7%, 21.6% and 8.3% five year survival for patients with a stage I, II, III, IV tumour respectively (Langdon, 1995).

· * NK; not known

(Edington et al., 1995; Prime et al., 1990).

## 3.3 Materials

LPA 16:0, LPA 17:0, LPA 18:0, LPA18:1, LPA 18:2 and LPA 20:4 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Plasma for standards were obtained from the University of Malaya Medical Centre (UMMC) blood bank. LPA was dissolved in ethanol:water (1:1), v/v to create a 10mM stock solution. Stock solutions were stored at -20°C. NS 398 was from Tocris Bioscience (Bristol, UK). Ki 16425 was from R&D Systems (Minneapolis, USA).

## 3.4 Cell Culture

### 3.4.1 Maintenance Of Cell Lines

All OSCC cell lines were cultured and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% foetal bovine serum (FBS), 200IU/mL penicillin, 200ug streptomycin and 0.15% hydrocortisone in an a $CO_2$ incubator (Binder Inc, NY, USA) with 5% $CO_2$ at 37°C (Chan et al., 1999; Edington et al., 1995; Prime et al., 1990).

All cell culture reagents and mediums were obtained from Gibco (Thermo Fisher Scientific Inc, MA, USA).

### 3.4.2 Sub-culture And Cell Number Determination

Cells were passaged at approximately 70% confluence. Following washing, cells were washed with phosphate buffered saline (PBS) and then incubated with 4mL trypsin/EDTA (Thermo Fisher Scientific Inc, MA, USA) for 20 minutes at 37°C. Trypsinised cells were resuspended in 4mL DMEM/F12 medium with 10% (v/v) fetal bovine serum (FBS) in a universal tube and then centrifuged at 1,000 rpm using Rotofix

32A (Hettich Instruments, LP. Tuttlingen, Germany) for 10 minutes. Cell pellets were resuspended in fresh media and cell numbers were determined. Cell suspensions were mixed 1:1 with Trypan Blue (Sigma-Aldrich Ltd., Gillingham, UK) and 10uLof the mixture pipetted onto Luna cell counting slide (Logos Biosystems Ins., Gyungyi-Do, Korea). The glass slides were inserted into the Luna Automated Cell Counter (Logos Biosystems Ins., Gyungyi-Do, Korea) for cell counting.

### 3.4.3 Storage And Recovery Of Cells

Aliquots of approximately $1x10^6$ cells were resuspended in FBS with 10% (v/v) DMSO (Sigma-Aldrich Ltd., Gillingham, UK). Cells were transferred to cryopreservation tubes (Nunc® Cryo Tubes®., Sigma-Aldrich Ltd., Gillingham, UK) and cooled slowly overnight in -80°C in a cryo container (Nalgen® Mr Frosty; Sigma-Aldrich Ltd., Gillingham, UK). Cells were subsequently stored in liquid nitrogen for long term storage.

To revive the cryopreserved cells, cryo vials were rapidly thawed at 37°C to minimize exposure to DMSO. Thawed cells were re-suspended in 5mL medium and centrifuged at 1,000 rpm for 10 minutes. Cells were transferred to a $25cm^2$ tissue culture flask in 5mL medium. The cells were passaged at least once before using them in any assays.

## 3.5 Assays Of Cell Behaviour

### 3.5.1 Transwell Migration Assay

Polycarbonate filters (8µM pore size; Transwell, Corning, USA) were coated with fibronectin (10 µg/mL) in 24-well plates (Costar, Corning, USA) for two hours at 37°C. Cells were cultured in reduced serum (DMEM/F12/1% FBS) overnight and treated with 10 µg/mL mitomycin C for two hours to inhibit cell proliferation. $0.5x10^6$ cells in media with reduced serum containing 0.25mg/mL fatty acid-free human albumin (FAFA) were seeded in the upper chamber. LPA and inhibitors were prepared in the same migration

buffer and placed in the lower chamber. Cells were allowed to migrate for 20 hours. Non-migrated cells were removed using cotton buds and migrated cells were rinsed with phosphate buffered saline (PBS) and stained with 0.1% crystal violet (in 20% methanol) for 2.5hours and counted in five random fields for each inserts under 20x magnification. All experiments were performed in triplicate and independent experiments were repeated at least twice.

### 3.5.2 Transwell Invasion Assay

Transwell invasion assays were carried out using Corning Biocoat Matrigel Invasion chambers with reduced growth factors (BD Biosciences, CA, USA). The invasion chambers contained 8μm pore size PET membrane with a thin layer of Matrigel Basement Membrane Matrix. The Matrigel Matrix is composed of laminin, collagen IV, nidogen/enactin and proteoglycan that mimics the basement membrane.

Following a 24-hour serum starvation and 2-hour Mitomycin C treatment, cells were collected using TrypLE Express (Thermo Fisher Scientific, Waltham, MA, USA) and $1x10^5$ cells were resuspended in 500μL of migration buffer. The cells were plated on top of the chambers and 750μL of migration buffer with LPA or inhibitors was added to the bottom compartment of the chamber. After 48 hours of incubation, the non-invaded cells on the upper surface of the membrane were removed with a cotton swab and the bottom chamber containing the invaded cells were stained and counted as described in the transwell migration assay (Section 3.5.1).

### 3.5.3 Cell Viability Assay

Cells were grown until 60-70% confluency. $1x10^4$ cells were seeded in each well in 96-well plates (Costar, Washington DC, USA) and cultured at 37°C for 24 hours. Cells were then treated with selected drugs which were also incubated according to respective time point (24, 48 and 72 hours accordingly). 10μL of 5mg/mL 3-(4, 5-dimethylthiazol-

2-yl)-2, 5-diphenyl tetrasodium bromide (MTT; Calbiochem, Merck Millipore) in PBS were added to each well and incubated for four hours. 150µL of 100% DMSO were added to each well to dissolve the formazan crystals formed. The plate was shaken for three minutes before measuring the absorbance at 575nm with a reference wavelength at 650nm using a micro plate reader (Infinite® 200 PRO, Tecan Trading, AG, Switzerland). The number of viable cells is directly proportional to the level of the formazan produced.

### 3.6 Radiation, LPA and Ki16425 treatment

OSCC cells were cultured and seeded in 30mL at 200,000 cells/dish in a 60mm dish from a mastermix of cells. Cell were cultured overnight and were pretreated with 10 µM LPA for one hour before the radiation treatment. In another set of experiment, OSCC cell lines were pretreated with 10 µM LPA for one hour followed by 10 µM LPA plus 10 µM Ki16425 or 10 µM NS398 for a further one hour before irradiation. Media were changed prior to irradiating the cells with 0-8 gray (Gy) gamma irradiation using Gammacell 3000.

#### 3.6.1 Clonogenic Cell Survival Assay

Irradiated cells were harvested immediately using trypsinisation. Cell were counted and a specific number of cells (300 and 500cells) were plated in triplicate for clonogenic survival assays. The cells were incubated for 12 days with a change of media every four days and colonies stained with 0.1% (w/v) crystal violet. Colonies with >50cells were counted as one colony. After counting the clones, plating efficiency (PE) and survival fraction (SF) were calculated using the following equation:

$$PE = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times 100\%$$

$$SF = \frac{\text{PE after irradiation} \times 100\%}{\text{PE of control}}$$

One and two Gy were selected for this study as the percentage of SF were more than 50%.

## 3.7 Plasmid Preparation And Transfection

### 3.7.1 Transformation Of Competent *Escherichia coli (E.coli)*

A 50µL aliquot of Dh5-Alpha Competent *E.coli* cells was thawed on ice prior to the addition of 1µg of plasmid DNA. The competent cell/DNA mix was incubated on ice for 15 minutes, heat shocked in a water bath at 42°C for exactly 45 seconds and promptly placed on ice for a further 2 minutes. 250µL room temperature Terrific broth (TB) (Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells and the mixture was shaken (200rpm, one hour, 37°C) prior to plating on 1.5% (w/v) TB/agar plates containing carbenicillin (50µg/mL; (Thermo Fisher Scientific, Waltham, MA, USA). Cultures were incubated inverted at 37°C for 16 hours. Single bacterial colonies were then inoculated into 3mL TB medium containing 50µg/mL carbenicillin and incubated overnight on an orbital shaker at 37°C.

### 3.7.2 Purification of Plasmid DNA

Plasmid DNA was purified using the Plasmid Maxi Kit (QIAGEN Ltd, Manchester, UK). 250mL of sterile TB containing 50µg/mL carbenicillin was inoculated with 250 µL of previously prepared 5mL culture of *E.coli* and incubated, with agitation, overnight at 37°C. The resultant cell suspension was centrifuged (5,300Xg, 10 minutes, 4°C), the supernatant discarded and the pellet re-suspended in 10mL Buffer P1 containing 100µg/mL of RNAse A. 10mL of Buffer P2 was added and mixed thoroughly by inverting 6-8 times, and incubated at room temperature for 5 minutes. 10mL of chilled Buffer P3 was then added to the lysate and mixed immediately but gently by inverting 6-8 times. The lysate was poured into the barrel of a QIAfilter Maxi Cartridge and incubated at room temperature for 10 minutes. The cap from the cartridge outlet nozzle was then removed, a plunger was inserted into the cartridge and the cell lysate was filtered into a 50mL tube. 2.5mL Buffer ER was added to the filtered lysate and mixed by inverting the tube

approximately 10 times. Following incubation on ice for 30 minutes, the filtered lysate was applied into a QIAGEN-tip 500 that had been pre-equilibrated with 10ml Buffer QBT. The lysate was allowed to enter the resin by gravity flow prior to washing the tip with 30mL Buffer QC. Plasmid DNA was eluted with 15mL Buffer QN and precipitated by the addition of 0.7 volumes room-temperature isopropanol. After centrifugation at 5,300Xg for 30minutes at 4°C, the supernatant was carefully decanted and the DNA pellet was washed with 5mL 70% (v/v) ethanol and centrifuged (5,3000Xg, 10 minutes). The DNA pellet was air-dried for 10 minutes at room temperature and dissolved in 500µL of Buffer TE.

### 3.7.3 RNA Interference

Silencing of *LPAR3* expression was achieved using short hairpin RNAs (shRNAs) delivered by lentiviral transduction to selected OSCC cell lines. Mission shRNA (Sigma Aldrich), scrambled or empty vector plasmids were added together with pMD2.G and psPAX2 plasmids to produce lentivirus in 293T cells. Mission shRNA plasmids are sequence-verified shRNA lentiviral plasmids for human genes for example *LPAR3* which have been cloned into pLKO.1-puro vectors. The shRNAs are processed into siRNAs intracellularly and mediate gene specific RNA interference (RNAi) in mammalian cells. The negative control for this shRNA experiment is the empty vector plasmid containing no shRNA insert and the scrambled plasmid is the vector with non-targeting shRNA that will activate the RNA-inducing silencing complex (RISC) and the RNAi pathway, but not targeting any human genes. psPAX2 is a packaging plasmid that is the backbone of the virus system. It has elements that are required for vector packaging such as structural proteins and enzymes that generate vector particles without any packaging signals to prevent virulence replication. pMD2.G is a VSV-G envelope expressing plasmid which is required to provide stability to the vector by bringing together the particles made by

the packaging plasmids. Co-transfection of all plasmids in a packaging cell for example 293T cells allows efficient production of lentiviral particles which are released into the cell supernatant (viral stocks). Viral stocks were stored at -80°C until further used. Selected OSCC cell lines, H357 and BICR31, were cultured in T25cm$^2$ until 50-60% confluence. Viral stock was diluted (1:1) with medium together with 8ug/mL of polybrene before being transduced to H357 and BICR31 separately. After 18hours/overnight incubation, media were changed and further incubated for 48hours. Puromycin (1 µg/mL) was used to select for stably transduced cells. Cells were cultured for several passages prior to use in any experiments. Knockdown expression of LPAR3 protein was confirmed using SDS-PAGE and western blots. The LPAR3 protein was detected using specific primary anti-LPAR3 and its secondary antibodies.

### 3.8 RNA Analysis

Prior to any experiments related to RNA, all work surfaces were decontaminated using RNAseZap® Decontamination Solution (Life Technologies Ltd, UK).

#### 3.8.1 RNA Extraction from Tissue Samples and Cell Lines

Total RNA extraction from tissues and cell lines was carried out using Qiagen® RNeasy® Mini kit according to the manufacturer's protocol (QIAGEN Ltd, Manchester, UK). Adherent cells were collected following trypsinisation (Section 3.4.2), washed once with ice cold PBS and the recommended volume of RLT buffer supplemented with β-mercaptoethanol (β-ME) was added and vortexed to homogenise the samples. For tissue samples. Approximately 30mg of snap-frozen biopsies of OSCC or normal oral mucosa were added with the RLT buffer with β-ME. 70% (v/v) alcohol was added to the lysates from cells or tissues and the mixture was transferred to RNAsey Mini spin column for

centrifugation. Subsequently, on-column DNAse digestion was performed according to the manufacturer's protocol using the RNAse-Free DNAse set (QIAGEN Ltd, Manchester, UK). Following multiple washes with the supplied buffer, RNA was eluted from the columnusing 40uL of RNAse-free water pipetted directly onto the membrane.The concentration and quality of RNA was measured using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA with a 260/280 ration close to 2 and a 260/230 ratio in the range of 2.0-2.2 were considered to be of acceptable purity and used for subsequent analysis.

### 3.8.2 Reverse Transcription (RT) of RNA to cDNA

One µg of RNA was subjected to reverse transcription using the High Capacity cDNA Reverse transcription kit (Applied Biosystems, California, USA). A stock solution containing 2uL of 10X RT Rndom Primers and 10X RT Buffer each as well as 0.8uL of 100mM deoxyribonucleotide triphosphates (dNTPs) and 3.2uL nuclease-free water was prepared and added to the RNA (10uL volume in sterile nuclease-free water).The sample was mixed and incubated using a VeritiR 96-Well Thermal cycer (Applied Biosystems, California, USA). Under the following conditions: 10 minutes at 25C, 2 hours at 37C, 5 minutes at 85C and hold at 4C. The cDNA samples were stored at -20C until further used.

### 3.8.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

QRT-PCR was performed using diluted cDNA (1:20 with nuclease-free water), in Fast Start Universal Probe Master (ROX; Roche Molecular Diagnostics, California USA) using the 7500 Fast Real-Time PCR system (Applied Biosystems, California, USA). The following TaqMan® Gene Expression Assay Probes ((*LPAR1*: Hs 00173500_m1; *LPAR2*: Hs 00173704_m1: *LPAR3*: Hs 00173857_m1; *LPAR4*: Hs 00271072_s1; *LPAR5*: Hs 00252675_s1; *LPAR6*: Hs 0027178_s1; ATX: Hs 00905125_m1 and *GAPDH* (4326317E) (Applied Biosystems, California, USA) was used separately to investigate

the expression levels of different mRNAs. *GAPDH* was amplified in the same reaction to serve as an internal control for normalization. Each reaction was prepared according to Table 3.5 in a 96-well optical reaction plates (Applied Biosystems®; Life Technologies Ltd, Paisley, UK). All samples were amplified in triplicates including a non-template control included in each run. The plates were sealed with optically clear adhesive film (Applied Biosystems®; Life Technologies Ltd, Paisley, UK) and centrifuged before placing it in the thermal cycler. The real-time cycler was programmed to the following standard relative quantification method: holding stage (2 minutes at 50°C followed by 10 minutes at 95°C) and cycling stage (15 seconds at 95°C followed by one minute at 60°C for 40 cycles).

Fold changes in gene expression were measured using the comparative threshold cycle method (ΔΔCt). Quantification of real-time PCR results analysis was performed using the 7500 Software version 2.0.5 system (Applied Biosystems, California, 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 (NE) values were calculated using the formula: $2^{-\Delta\Delta Ct}$.

**Table 3.5: Reaction mix for QPCR analysis**

| Component | Manufacturer | Volume per reaction (μL) |
|---|---|---|
| 20X Taqman Gene Expression Assay for target gene (contains FAM labelled primer/probe) | Thermo Fisher Scientific Inc. Waltham, MA, USA | 1 |
| 20X Taqman Gene Expression Assaay for *GAPDH* (contains VIC NFQ-MGB labelled primer/probe) | Thermo Fisher Scientific Inc. Waltham, MA, USA | 1 |
| 2X FastStart Universal Probe Master mix | Roche Diagnostics Limited, UK | 10 |
| Muclease-free water | Gibco®, Life Technologies Ltd, Paisley, UK | 3 |
| Diluted cDNA | N/A | 5 |
| | Total volume per reaction | 20 |

N/A – not available

## 3.9 Protein Analysis

### 3.9.1 Western Blotting

#### 3.9.1.1 Protein Extraction And Quantification

Cells were cultured in 100mm dishes, washed in ice-cold PBS and harvested either with cell scraper (Corning) of by trypsinisation (as described in Section 3.4.2). Cells were pelleted down by centrifugation. Cells were lysed in RIPA lysis buffer (0.15M NaCl, 1%(v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50mM Tris-HCl (pH 8.01)) with protease inhibitors (cocktail set III; Calbiochem, Merck Millipore, Darmstadt, Germany) and phosphatase inhibitors (Halt phosphatase inhibitor cocktail; Thermo Fisher Scientific, Waltham, MA, USA). The lysates were vortexed, incubated on ice for 30 minutes and later centrifuged at maximum speed (16,000Xg) for another 30

minutes at 4°C on a refrigerated centrifuge (Eppendof, Hamburg, Germany). Supernatant was transferred to a clean 06mL tube for immediate use or frozen at -80C.

Protein concentrations were estimated using Bradford assay (BioRad. CA, USA). Typically, protein lysates were diluted 10X (with PBS) before using them for protein estimation. Bovine Serum Albumin (BSA) standard set (BioRad. CA, USA) which consist of 7 concentrations of BSA (0-2mg/mL), was used to construct the standard curves. Briefly, 250uL of 1X Dye Reagent at room temperature was added to each well of a 96-well plate. 5uL of BSA standard or diluted protein samples were added separately into each well. Absorbance was measured at 595nm after shaking the pates for 3 minutes using a microplate reader (Infinite® 200 PRO, Tecan Trading, AG, Switzerland). A standard linear curve was constructed using the BSA standards and the concentrations of protein samples were estimated based on the curve.

#### 3.9.1.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared using 10% EZ-Run™ protein gel (Fisher Scientific, Walham, MA, USA). Gels were placed in Mini-PROTEAN ® Tetra Electrophoresis Systems (BioRad. CA, USA) filled with Running buffer (Table 3.6). A total of 50µg of protein lysate was added with equal volumes of 2X Laemmli sample buffer ((BioRad. CA, USA). The mixture was denatured by heating at 95C for 10minutes. Samples were loaded loaded into each well on the SDS-PAGE gels along with PrecisionPlus Protein Standard (All Blue; BioRad. CA, USA). Electrophoresis was performed at 70V for 20minutes and increased later to 100V until the end of the run.

**Table 3.6: Solutions for SDS-PAGE and Western blotting**

| Solutions | Amount |
|---|---|
| Running buffer | |
| 20X Running Buffer (Fisher BIOReagent, Thermo Fisher Scientific, MA,USA) | 50mL |
| Distilled water | 950mL |
| 10X Transfer buffer (1L) | |
| Tris salt (Merck Millipre Darmstadt, Germany) | 30.3g |
| Glycine (Merck Millipre Darmstadt, Germany) | 144.0g |
| Distilled water | 1L |
| 1X Transfer buffer (1L) | |
| 10X Transfer buffer (1L) | 100mL |
| Methanol | 200mL |
| Distilled water | 700mL |
| 10X TBS (1L) | |
| NaCl | 80g |
| Tris salt (Merck Millipre Darmstadt, Germany) | 24.2g |
| HCL (to adjust pK to 7.6) | Variable |
| Distilled water ( to top up to prepare 1L solution) | Variable |
| 1X TBST (1L) | |
| 10X TBS | 100mL |
| Tween-20 (Bio Basic Inc., Markham, Canada) | 1mL |
| Distilled water | 900mL |

HCL, hydrochloric acid; NaCl, sodium chloride, TBS, TRIS buffered saline.

### 3.9.1.3 Protein Transfer

After SDS-PAGE, proteins were transferred from the gel to the polyvinylidene diflouoride (PVDF) membranes (Immobilon-P, Millipore) using a semi-dry transfer system. Prior to the transfer, the membrane was activated by soaking in methanol for 15 seconds followed by 2 minute in ultrapure water and 5 minutes in 1X Transfer Buffer (Table 3.6). Protein transfer was performed using a Mini Transblot® Cell ((BioRad. CA, USA) at 100V for 90 minutes.

### 3.9.1.4 Immunoblotting And Visualisation

PVDF membranes were blocked for one hour at room temperature with either 5% (w/v) BSA or non-fat milk powder in TRIS-buffered saline (TBS) with 0.1% (v/v) Tween

20 (TBST). The membranes were incubated overnight with respective primary antibodies (Table 3.7) at 4°C. The membranes were washed three times with TBST, before incubation with respective secondary antibodies for one hour (Table 3.7).

Non-specific protein binding was blocked by incubating the membrane for one hour at room temperature in 5% non-fat dried milk of 5% bovine serum albumin (BSA; Bio Basic Inc., Markham, Canada) dissolved in TRIS buffered saline with 0.1% Tween 20 (TBST) (Table 3.6). Membranes were incubated with primary antibody (Table 3.7) dissolved in blocking solution at 4C. Following overnight incubation, membranes were washed three times in TBST before incubation for one hour at room temperature with the following corresponding horse-radish peroxides (HRP)-conjugated secondary antibodies dissolved in blocking solution. After washing 3 times in TBST, the membranes were incubated with Western Bright™ Sirius (Advansta, CA, USA) and chemiluminescence produced by protein-HRP-complex substrate was detected using Odyssey FC Imaging system (Licor Biosciences, Cambridge, UK).

**Table 3.7 Antibody details and dilutions.**

| Antibody | Manufacturer | Antibody dilution |
|---|---|---|
| Anti-alpha-tubulin | Sigma Aldrich | 1:10000 |
| Anti-beta-actin | Sigma Aldrich | 1:5000 |
| Anti-LPAR3 | Sigma Aldrich | 1:1000 |
| Anti-EGFR | Cell Signalling | 1:1000 |
| Anti-phosphoEGFR | Cell Signalling | 1:1000 |
| Anti-COX2 | Santa Cruz Biotechnology | 1:500 |
| Secondary donkey anti-goat antibody | Santa Cruz Biotechnology | 1:3333 |
| Secondary goat anti-rabbit antibody | Sigma Aldrich | 1:5000 |

### 3.10 Liquid Chromatography Mass Spectrometry (LC-MS/MS)

#### 3.10.1 Standard and Quality Control (QC) Samples For LC-MS/MS.

All LPA stocks and the internal standard (IS; LPA17:0) were prepared in 50% (v/v) ethanol each at a concentration of 1.0mg/mL, which is equivalent to 1000 parts per million (ppm).

A concentrated mixture of all LPA standards (working stocks) was prepared in 100% methanol. A diluted working stock of 2000ppb was finally prepared in blank plasma to prepare 100ppb, 200ppb, 350ppb, 500ppc, 700ppb, 1000ppb and 1500ppb as the standard curve for quantification. Quality control (QC) samples were prepared at 300ppb (QC1), 750ppb (QC2) and 1350ppb (QC3). Calibration standards and QC samples were prepared fresh daily. LPA 17:0 was prepared as a stock of 1ppm separately for use as an internal standard (IS). Blank plasma was prepared using expired plasma from the blood bank. 10% (w/v) of activated charcoal was added to the plasma and left shaking overnight. Plasma was centrifuged at 16000Xg for 10mins. Supernatant was filtered using 0.22μM nylon filter. After treatment, supernatant was analysed for LPA species. If no LPA was detected, then the blank plasma is really to be used. . If LPA was detected, the charcoal treatment was repeated again. Following which the sample was tested again for LPA. This cycle was continued for nine days. Plasma was also treated by freezing for one hour and thawing, twice in day-8 and day-9.

#### 3.10.2 Plasma Sample Collection

Three mL of whole blood was collected in EDTA tube and kept on ice. Samples were centrifuged at 300xg for 15mins, aliquoted (300uL/aliquot) and stored at-80°C until further use.

### 3.10.3 Sample Preparation for LC-MS/MS

20µl of IS was spiked into 10ul of standard mixture or plasma samples. The lysophospholipid fraction was extracted using a modified and simple method using methanol. Briefly, 300ul of 100% methanol was added to the mixture and vortexed for one minute. The sample was then incubated for 10mins on ice and later centrifuged at 16000g for 10mins. Supernatants were removed and transferred to a fresh glass vial and subjected to LC-MS/MS analysis.

### 3.10.4 LC-MS/MS

The LPA species were separated by reversed-phase liquid chromatography using a C6 Phenyl analytical column (Gemini, particle size 5 µm, pore size 110Å, 150 x 2.0mm, Phenomenex, Torrance, CA, USA) and an injection volume of 10uL with a 10min separation time. The mobile phase consisted of $H_20$ containing 0.01% $NH_3$ and Acetonitrile: Methanol (50:50, v/v) and was delivered at a flow rate of 0.5mL/min. The LC-MS/MS system comprised of an Applied Bioscience (AB) SCIEX QTRAP 5500 mass spectrometer, equipped with a Shimadzu LC system and auto sampler. The electrospray ion source was run in a negative ionization mode. Quantification of LPA was performed using a triple quadrupole LC-MS/MS in the multiple reaction-monitoring (MRM) mode. Calibration standards as stated above and QC samples were extracted and quantitated daily and approximately 20-30 plasma samples were analysed daily. Quantitative data measured against a linear standard curve was analysed with Analyst data acquisition system.

### 3.10.5 Method Validation

Intra-day or inter-day precision and accuracy (n=5) were evaluated by spiking a known amount of LPA and IS at three different concentrations, namely, 300ppb(QC1), 750ppb(QC2) and 1350ppb(QC3). Freeze and thaw stability were tested with QC1 and

QC3 where all samples were kept frozen at -40C for 25mins and thawed at room temperature for three cycles. Analysis was carried out for every cycle. Long term stability of QC samples was analysed after 48days.

## 3.11 Statistical Analysis

### 3.11.1 Statistical Tests

All statistical analysis were performed using Graph Pad Prism Version 5.01 and SPSS Version 20. Parametric tests such as one-way ANOVA ($p<0.05$) were performed when the samples were normally distributed, whilst a nonparametric test (Mann-Whitney U test; $p<0.05$) was performed in circumstances when samples were not normally distributed. Gene expression correlations were analysed using Spearman correlation test ($p<0.05$). The associations of gene expression with the clinicopathological characteristics of the samples were analysed using Pearson chi-square test ($p<0.05$). Differences between the three sample groups were analysed using Kruskal-Wallis post hoc test ($p<0.05$). Scatter plots and principle component analysis were analysed using the R software version 3.2.5.

# CHAPTER 4:

# ROLE OF LPA AND ITS RECEPTORS IN OSCC.

## 4.1 Introduction

For more than a decade, LPA signalling has been implicated in cancer and there is mounting evidence that suggest crucial roles for the LPA-LPAR-axis in cancer progression and metastasis. For example, aberrant LPA production, receptor expression, and signalling are thought to contribute to cancer initiation, progression and metastasis. *In vitro* experiments have shown that LPA can stimulate cell growth and proliferation (Goldsmith et al., 2011; Hurst et al., 2009; Leve et al., 2015; Xu et al., 1995), motility (Fukushima et al., 2018; Liao et al., 2013; Shida et al., 2003; Yu et al., 2008) invasion (Hayashi et al., 2011; Hwang et al., 2016; Okabe et al., 2011) and resistance to chemo-radiotherapy (Deng et al., 2007; Schneider et al., 2014), whilst reducing LPA levels by increasing LPA hydrolysis and decreasing enzymes that produce LPA *in vivo* can inhibit tumour progression (Tanyi et al., 2003). In patients, LPA levels have been reported to be markedly elevated in biological fluids such as the ascites fluid of ovarian cancer patients and the blood of other gynaecological cancers (Xu et al., 1998; Yoon et al., 2003) and multiple myeloma (Sasagawa, Okita, et al., 1999).

In a comprehensive transcriptome analysis of published microarrays from more than 350 normal tissues and 1717 malignant tissues from 16 different cancers), LPARs were shown to be highly expressed in several human tumour types; different cancers display distinct expression signatures of LPARs that distinguish them from one another (Willier et al., 2013). The expression of the LPARs in oral cancer, however, has not been examined.

Another important contributor to this signalling axis is the enzyme autotaxin (ATX), the protein product of the *ENPP2* gene. ATX converts lysophosphatidylcholine (LPC)

into LPA by hydrolysing the choline head group. It was first identified as a tumour motility-stimulating protein, isolated from the conditioned medium of melanoma cells, A2058 (Stracke et al., 1992). About a decade later, ATX was linked to LPA production and was shown to be a major producer of circulating LPA levels; LPA levels are reduced by approximately 50% in *ENPP2* knockout mice (Umezu-Goto et al., 2002). ATX is highly expressed in a variety of carcinomas and sarcomas (Willier et al., 2013), but its expression in OSCCs is currently unknown. However, ATX mRNA has been reported to be upregulated in 46% (7/15) of micro-dissected NPCs, a sub-group of head and neck cancer (Yap et al., 2015).

In summary, there is mounting evidence suggesting that LPA signalling drives the process of carcinogenesis and radiotherapy resistance. However, very little is known about its role in OSCC. The aim of this study, therefore, was to examine the expression of ATX and LPARs in OSCC cell lines and tissues and to investigate the role of this pathway in regulating tumour cell migration/invasion and the response to radiation *in vitro*.

## 4.2 Results

### 4.2.1 Expression of *ENPP2* in normal oral and OSCC tissues.

ATX is the enzyme that produces LPA and it is the protein product of the gene *ENPP2*. In this study, the expression of the *ENPP2* gene was examined using quantitative real-time PCR (qRT-PCR) analysis in five normal and 52 OSCC tissues. There was no significant difference in the expression of *ENPP2* between normal and tumour samples, although the level of expression in OSCC tissues was more variable than in normal mucosa (Figure 4.1). Figure 4.2 shows the distribution pattern of *ENPP2* gene expression in the same group of samples. Interestingly, a subset of tumour samples overexpressed the *ENPP2* gene.

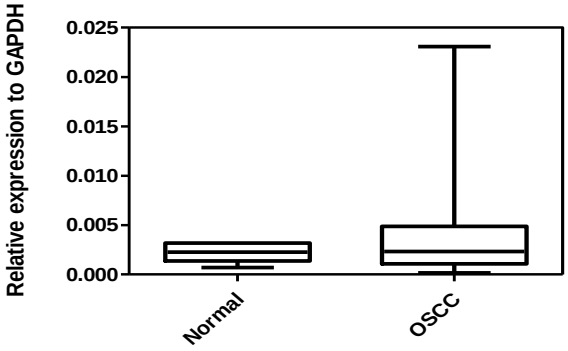



**Figure 4.1: Expression of *ENPP2* in normal and OSCC tissues.**
The expression of *ENPP2* was examined in 52 tissues of OSCC patients and five normal oral mucosa samples by qRT-PCR. Box-plot shows mRNA expression of *ENPP2* gene (normalized to *GAPDH*) in the tissue samples of normal oral mucosa and OSCCs. There was no significant difference in the expression of *ENPP2* in OSCC tissues as compared to normal. (* indicates $p < 0.05$ in Mann Whitney U test).

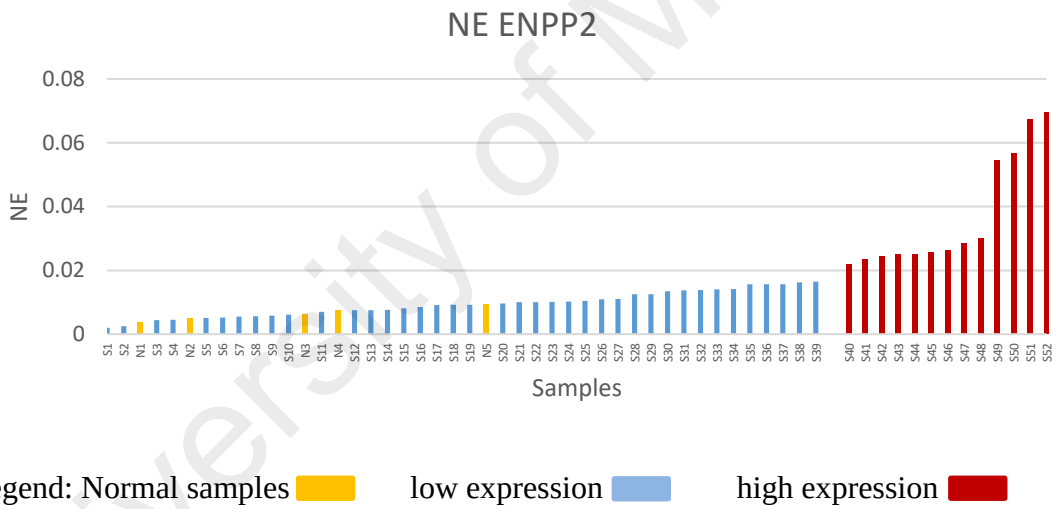



**Figure 4.2: Distribution of *ENPP2* expression in normal and OSCC tissue.**
The mRNA expression levels of *ENPP2* in normal samples were all classified as low (orange bars). On the other hand, in OSCC samples, they can be classified into two groups: low and high (blue and red bars respectively). Normalised expression (NE) values for every tissue samples were obtained from qPCR analysis ($NE = 2^{-\Delta\Delta CT}$). Values lower than the median NE were assumed to have low expression (blue bar) whereas those with higher than median NE were considered to have high expression (red bar). Normal samples are indicated as orange bars.

### 4.2.2 Expression of LPA receptors in OSCC cell lines.

To begin to investigate LPAR expression in OSCC, the mRNA levels of *LPAR 1-6* were first measured in eight OSCC cell lines by qRT-PCR (Figure 4.3). All *LPARs* except for *LPAR4* were expressed in all cell lines analysed. Only H376 did not express *LPAR4*.

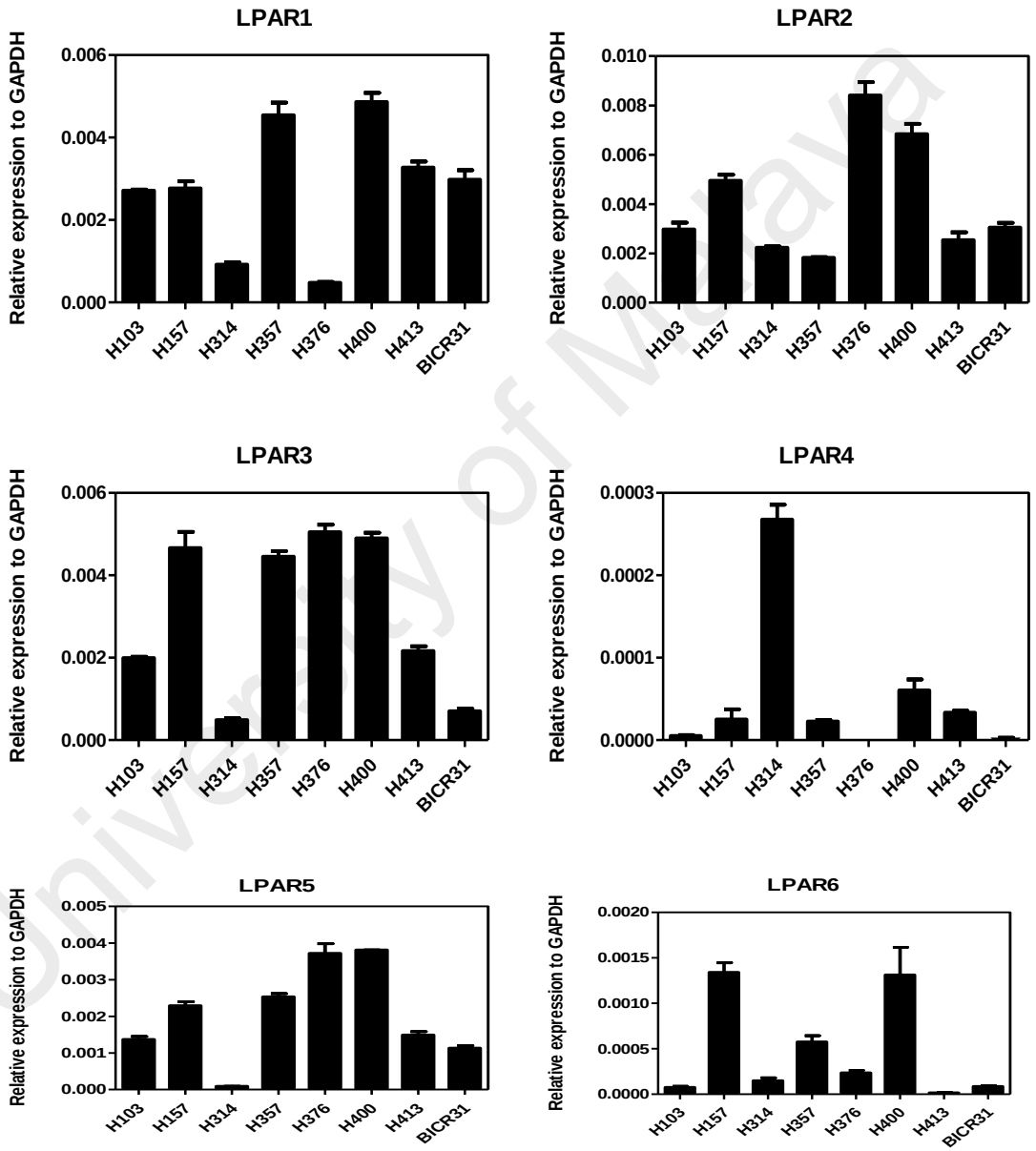



**Figure 4.3: Expression of *LPAR*s in OSCC cell lines.** Graphs show the mRNA expression of the indicated genes (normalized to *GAPDH*) in eight OSCC (H103, H157, H314, H357, H376, H400, H413 and BICR31, all in black bars) by qRT-PCR. Data represents the results from at least two independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates.

### 4.2.3 Expression of *LPAR3* in normal oral and OSCC tissues

A number of research has been carried out focusing on LPA signalling in cancer. However, the majority of the work focused on *LPAR1* and *LPAR2*. Limited work has been done with regards to LPA signalling in HNSCC particularly on OSCC. Therefore, this study chose to focus on *LPAR3* in OSCC. The expression of *LPAR3* was examined in 52 OSCCs and 5 control tissues by qRT-PCR analysis. The expression of *LPAR3* was significantly higher in OSCC tissues as compared to normal tissues ($p<0.05$, Figure 4.4).

(A)

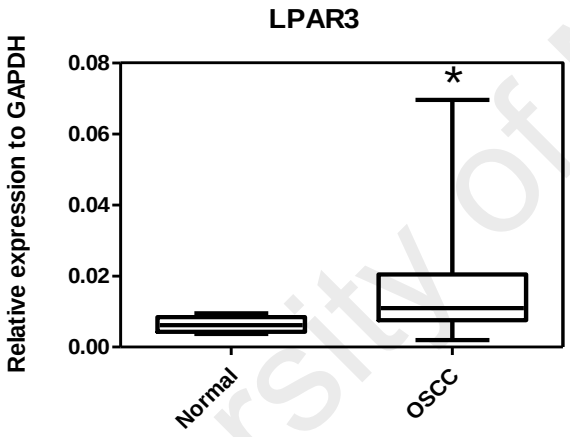
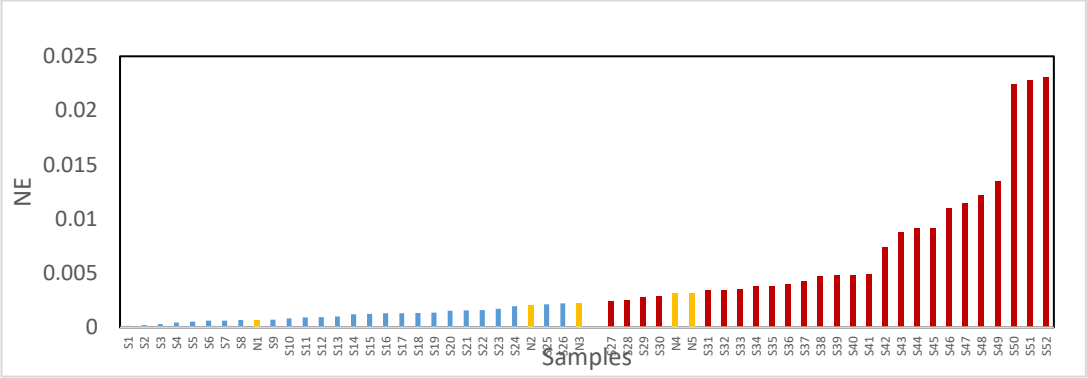



**Figure 4.4: *LPAR3* gene expression in normal and OSCC tissues.**
The expression of *LPAR3* gene was examined in 52 tissues of OSCC patients and five normal oral mucosa samples by qRT-PCR. Box-plot shows mRNA expression of *ENPP2* gene (normalized to *GAPDH*) in the tissue samples of normal oral mucosa and OSCCs. There was a significant upregulation of *LPAR3* gene expression in OSCC tissues compared to normal tissues. (* indicates $p < 0.05$ in Mann Whitney U test).

Figures 4.5 shows the distributions of *LPAR3* gene expression from all the tissue samples. Samples were divided into two groups i.e. high and low expression (compared to the median value). For *LPAR3*, approximately 50% of cases fell into each group, demonstrating that nearly half of all OSCC tumours over-expressed *LPAR3*.




Legend: Normal samples low expression high expression

**Figure 4.5: Distribution of *LPAR3* gene expression in normal and OSCC tissue.** The mRNA expression levels of *LPAR3* of normal samples were classified in both groups low and high (orange bars). On the other hand, half of OSCC samples fell into the low expression group and the other half were classified as having high expression (blue and red bars respectively). Normalised expression (NE) values for every tissue samples were obtained from qPCR analysis ($NE = 2^{-\Delta\Delta CT}$). Values lower than the median NE were assumed to have low expression (blue bar) whereas those with higher than median NE were considered to have high expression (red bar). Normal samples are indicated as orange bars.

### 4.2.4 Correlation between *LPAR3* and *ENPP2*.

To study the relationship between the levels of individual gene expression, the correlation between the expression of *LPAR3* and *ENPP2* was analysed using Spearman rank correlation test. The results indicated a positive but low correlation between *LPAR3* and *ENPP2* gene expression (Table 4.1; Figure 4.6).

**Table 4.1: Positive correlation between *LPAR3* and *ENPP2* gene expression**. Spearman correlation test was performed for the two genes (normalised against *GAPDH*). The table shows the correlation between the two genes (N=52). A positive and low correlation was observed (with p=0.008) where expression of *LPAR3* increases coincidentally with *ENPP2* (* indicates p<0.05, ** p <0.01, *** p <0.001).

| Variables | *LPAR3* | *ENPP2* |
|---|---|---|
| *1. LPAR3* | - | .367** |
| *2. ENPP2* | .367** | - |

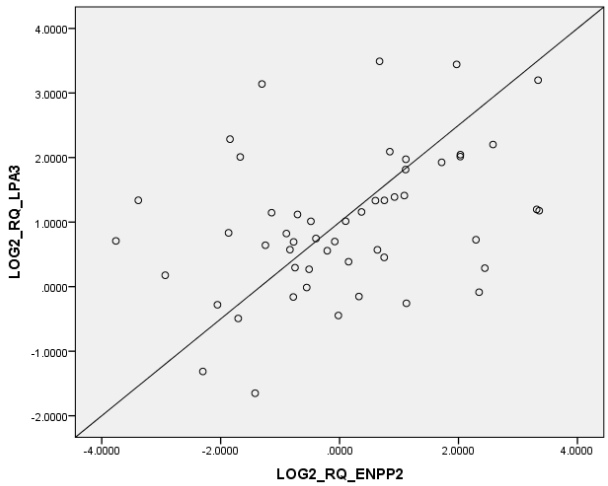



**Figure 4.6: Scatter plot showing positive correlation between *LPAR3* and *ENPP2* gene expression.**
The plot was generated using the Log2 RQ values of *ENPP2* and *LPAR3* from the samples.

### 4.2.5 Association of *LPAR3* and *ENPP2* expression with clinicopathological characteristics of OSCC patients.

To determine whether the expression of *LPAR3* and *ENPP2* was associated with any aetiological or clinical parameters, Pearson's Chi square tests were performed. The expression of both *LPAR3* and *ENPP2* had a signification association with betel-nut chewing status of the OSCC patients ($p<0.05$ (Table 4.2). The expression of *ENPP2* was associated with cancer stage. No associations were found for smoking and lymph node metastasis status of the patients.

**Table 4.2: Independent association of *LPAR3* and *ENPP2* genes with smoking, betel-nut chewing, cancer stages and lymph nodes metastasis (LNM) of OSCC patients.**
Pearson's chi square test was carried out to determine the association of each gene expression with the risk factors and tumour characteristics of OSCC patients (* indicates $p<0.05$).

| Genes | Smoking | Betel-Nut chewing | Cancer stage | LNM status |
|---|---|---|---|---|
| ***LPAR3*** | 0.313 | 0.034* | 0.868 | 0.337 |
| ***ENPP2*** | 0.08 | 0.048* | 0.044* | 0.579 |

### 4.2.6 Effects of LPA on the migration and invasion of OSCC cells.

The effect of LPA on cell migration was examined using two OSCC cell lines, H357 and H376 and transwell cell migration assays. LPA enhanced the migration of H357 cells when used at 10µM (Figure 4.7A); lower concentrations did not induce cell migration (data not shown). The migration of H376 cells was increased by all concentrations tested (2.5 - 10µM Figure 4.7B). To standardize future migration assays, 10 µM of LPA was chosen for both cell lines.

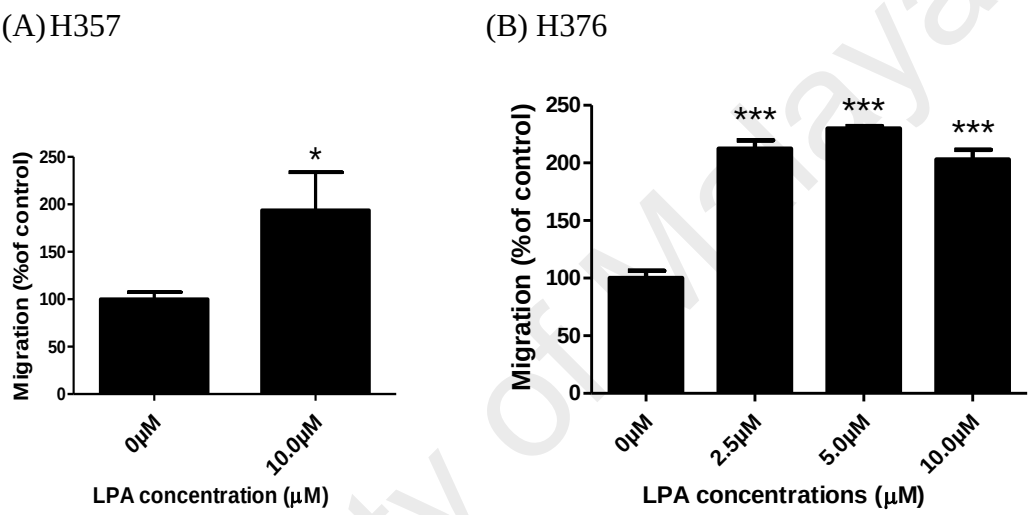



**Figure 4.7: LPA induced cell migration.**
(A)H357 and (B) H376 were cultured in reduced serum media (1%FBS) for 24 hours. The cell lines were first treated with mitomycin C (10ug/mL) for two hours, followed by treatment with vehicle control (50% Ethanol). Treatment with various LPA concentrations (2.5, 5.0 and 10 µM) were carried out for 18 hours in the transwell migration assay. Cells were washed with PBS and stained with 0.1% crystal violet in methanol for 2.5hours. Bars, mean; error bars, standard deviation (SD). (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in T test and Dunnett's test).

### 4.2.7 Role of LPAR1/3 in OSCC cell migration and invasion.

To begin to study the role of specific LPA receptors in regulating cell migration, the effect of Ki16425, an inhibitor of LPAR1 and LPAR3, on the migration of H357 and H376 cells was examined. Ki16425 has been reported to inhibit LPAR1 and LPAR3 in various cell types, for example A431 (epithelial carcinoma), PC12 (rat with

pheochromacytoma) and THP-1 (acute monocytic leukemia) (Ohta et al., 2003). MTT assays were first performed to assess the cytotoxicity of Ki16425. Limited toxicity was observed in both cell lines H357 and H376 treated with increasing concentrations of Ki16425 up to 50 µM for 24 and 48 hours (Figure 4.8). Ki16425 has been reported to efficiently inhibit LPAR1 and LPAR3 at 10 µM (Ohta et al., 2003), therefore this concentration was used in subsequent experiments to examine the effect of these receptors in cell migration.

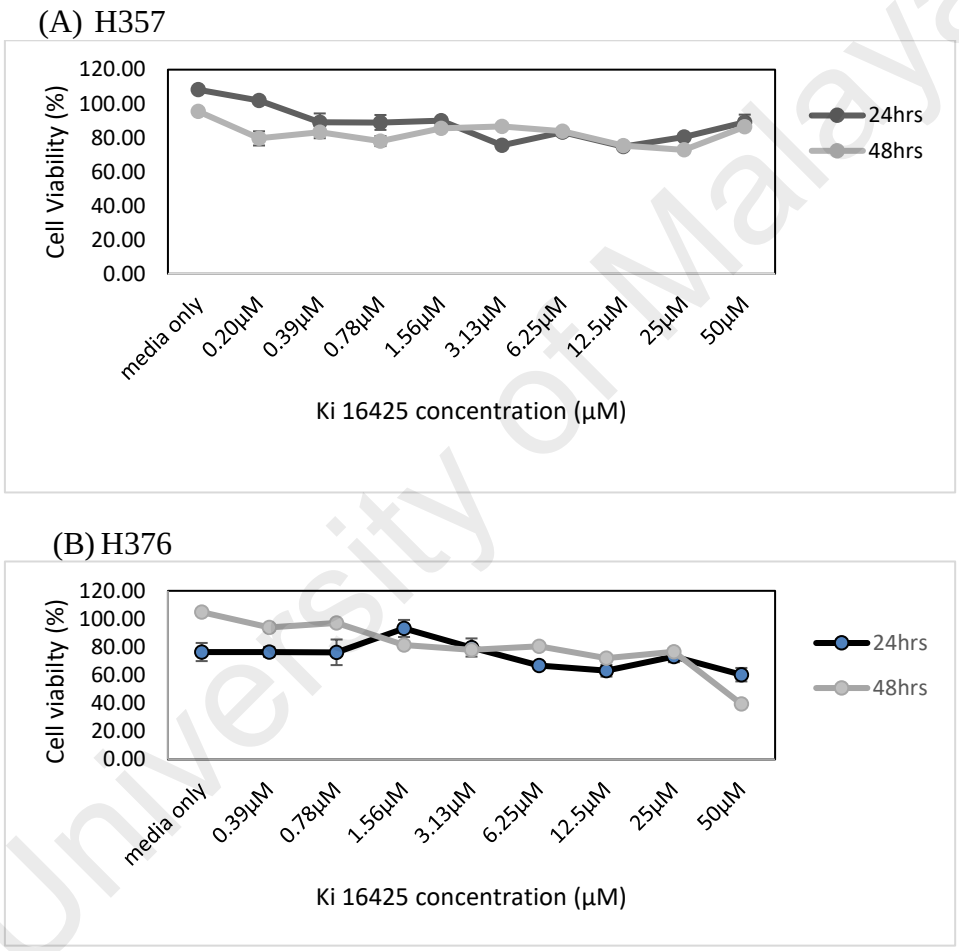



**Figure 4.8: Effect of Ki16425 on cell viability of H357 and H376.**
MTT assays were performed on (A) H357 and (B) H376 treated with increasing concentration of Ki16425 for 24 and 48 hours. Graph shows the percentage of cell viability expressed in relative to the vehicle control. Data, mean; error bars, standard deviation.

LPA-induced cell migration was significantly attenuated by 10μM Ki16425 in both cell lines (Figure 4.9). Ki16425 also inhibited the invasion of H357 cells in Matrigel invasion assays *in vitro* to (Figure 4.10).These data demonstrate that activation of LPAR1 and/or LPAR3 is important for LPA-induced migration/invasion of OSCC cells.

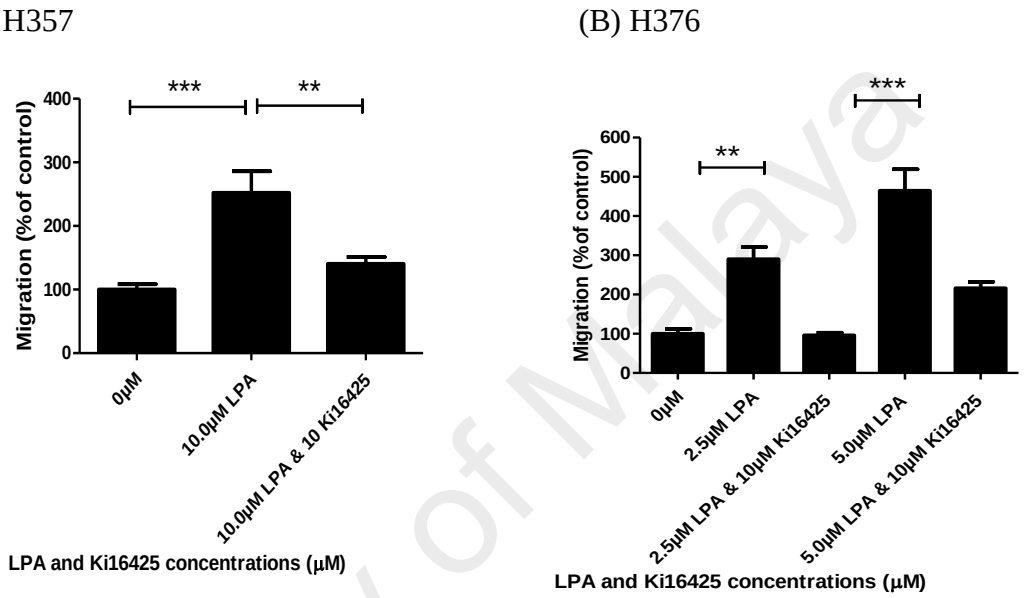



**Figure 4.9: Inhibition of LPAR1/3 reduces LPA-induced OSCC cell migration.** H357 and H376 were cultured in media with reduced serum (1%FBS) for 24 hours. The cell lines were then treated with mitomycin C (10ug/mL). Treatment with vehicle control (50% Ethanol), various LPA concentrations (2.5, 5.0 and 10μM) or combination of both LPA and 10μM Ki16425 were carry out for 18hours in transwell migration assay. Cells were washed with PBS and stained with 0.1% crystal violet in methanol for 2.5hours. Bars, mean; error bars, standard deviation (SD). (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

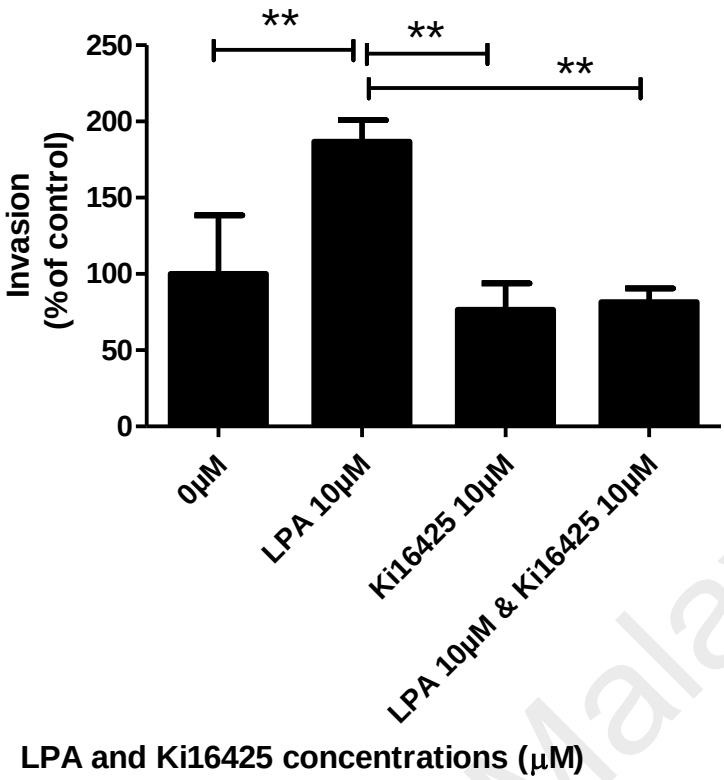



**Figure 4.10: Inhibition of LPAR1/3 reduces LPA-induced invasion.**
H357 was cultured in media with reduced serum (1%FBS) for 24 hours. The cell lines were then treated with mitomycin C (10ug/mL) and later with vehicle control (50% Ethanol), LPA (10μM), Ki16425 (10μM) or a combination of both 10μM LPA and 10μM Ki16425. Treatments were carry out for 48hours and the cells were quantitated by washing with PBS and stained with 0.1% crystal violet in methanol for 2.5hours. Bars, mean; error bars, standard deviation (SD). (ns indicates not significant, * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

### 4.2.8 Expression of LPAR3 in OSCC cell lines.

To further study the function of LPAR3 in the signalling axis, protein levels for LPAR3 were examined in the same panel of OSCC cell lines using western blotting (Figure 4.11). All OSCC cells expressed LPAR3. There was some heterogeneity in the level of expression with the highest expression observed in H103, H357, BICR31 and followed by moderate level of LPAR3 expression from H314 and H376 cells. H357, H376 and BICR31 were selected for a more detailed study focusing on LPAR3.

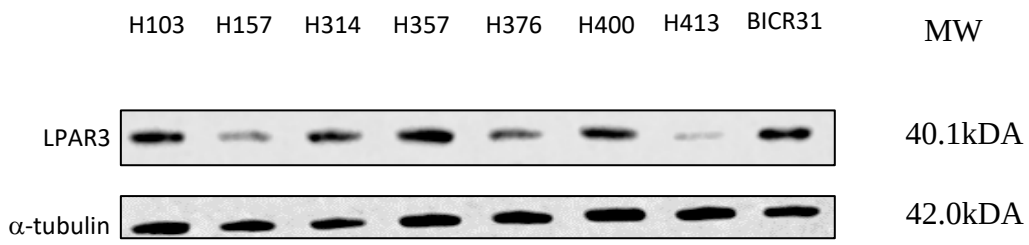



**Figure 4.11: Protein expression of LPAR3 in a panel of OSCC cell lines (H103, H157, H314, H357, H376, H400, H413 and BICR31).**
Highest protein level was observed in H103, H357 and BICR31. Blots are representative of three other experiments.

### 4.2.9 Effect of shRNA-mediated depletion of *LPAR3* on OSCC cell migration

To specifically investigate the role of LPAR3 in OSCC cell migration, the effect of silencing the expression of *LPAR3* using lentiviral vectors expressing *LPAR3* shRNA sequences was investigated. Based on the baseline protein levels of LPAR3 in the panel of OSCC cell lines (Figure 4.12), H357 and BICR31 were selected for the knockdown experiments. H357 and BICR31 were stably transduced with two sequences of *LPAR3* shRNA (designated sh90 and sh91). The control included cells transfected with a non-specific shRNA sequence (NS). Validation of *LPAR3* knockdown was shown by downregulation of both mRNA and protein levels in H357 and BICR31 (Figure 4.12).

*LPAR3* knockdown did not affect the proliferation of H357 and BICR31 cells (Figure 4.13 (A) and (B)).

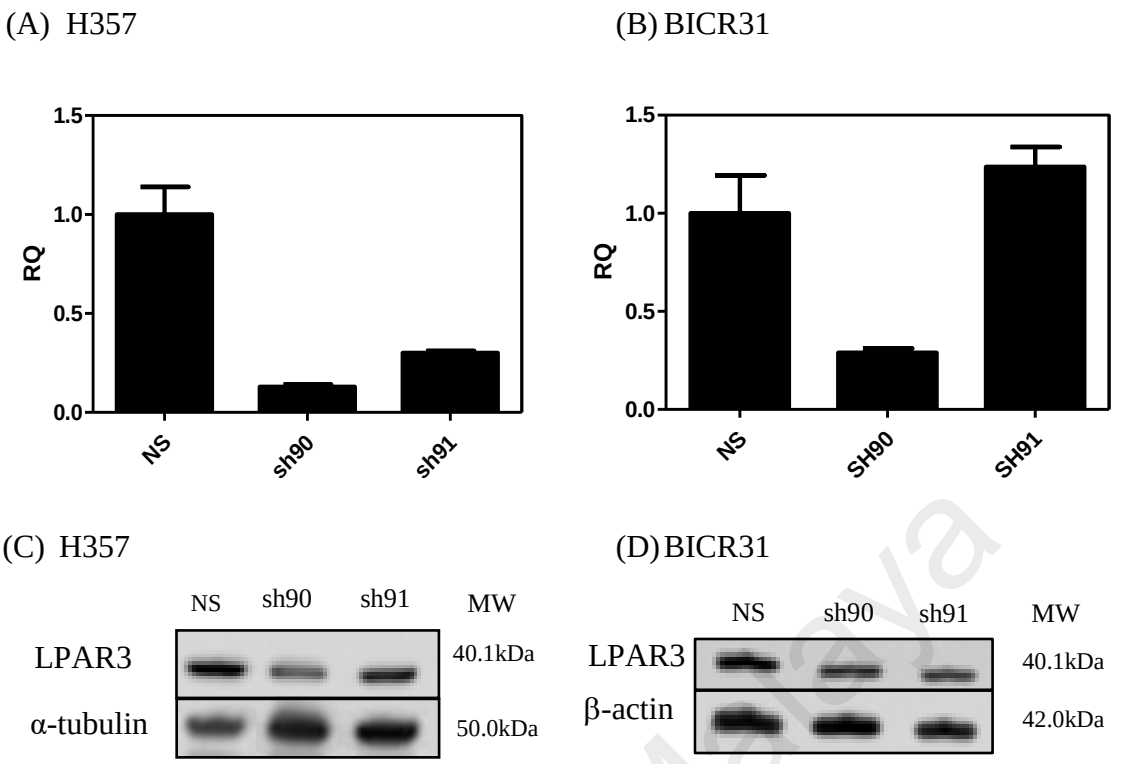



**Figure 4.12: Knockdown of LPAR3 in H357 and BICR31.**
*LPAR3* mRNA expression levels were reduced in knockdown cell lines of H357 (A) and BICR31 (B). In H357, there was a reduction of 87% in sh90 and 70% in sh91. In BICR31, only sh90 showed a reduction of 71% of *LPAR3* mRNA levels as compared to NS. LPAR3 protein expression levels were also shown to be reduced in both cell lines H357 (C) and BICR31 (D).

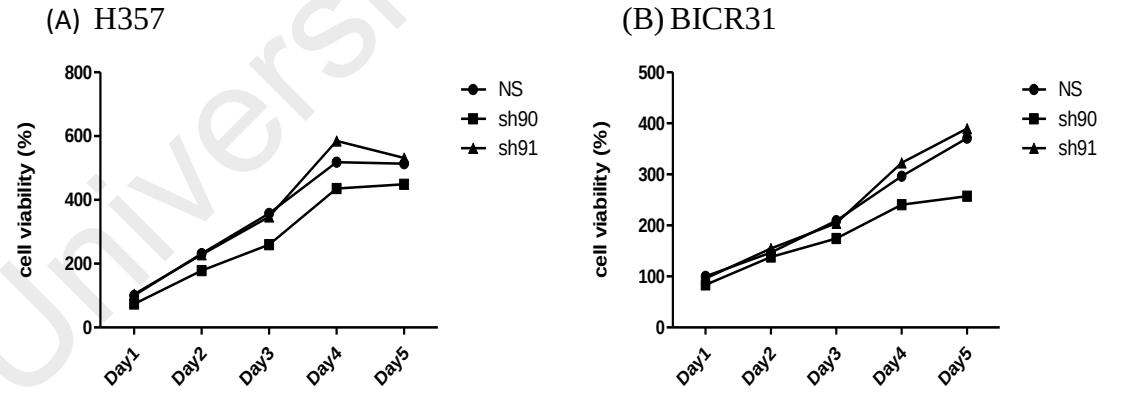



**Figure 4.13: Knockdown of LPAR3 did not inhibit OSCC cell proliferation.**
MTT assays were performed on (A) H357 and (B) BICR31 LPAR3 knockdown cell lines. Results represents the percentage of viable cells relative to complete medium for five consecutive days. Graphs are representative of at least three experiments. Data points, mean; error bars, SD of triplicates.

The effect of silencing the gene expression of *LPAR3* in H357 and BICR31 cells on cell migration was examined using transwell migration assays. LPA-induced cell migration was significantly attenuated in both cell lines transfected with both shRNA sequences (sh90 and sh91; Figure 4.14).

(A) H357 (B) BICR31

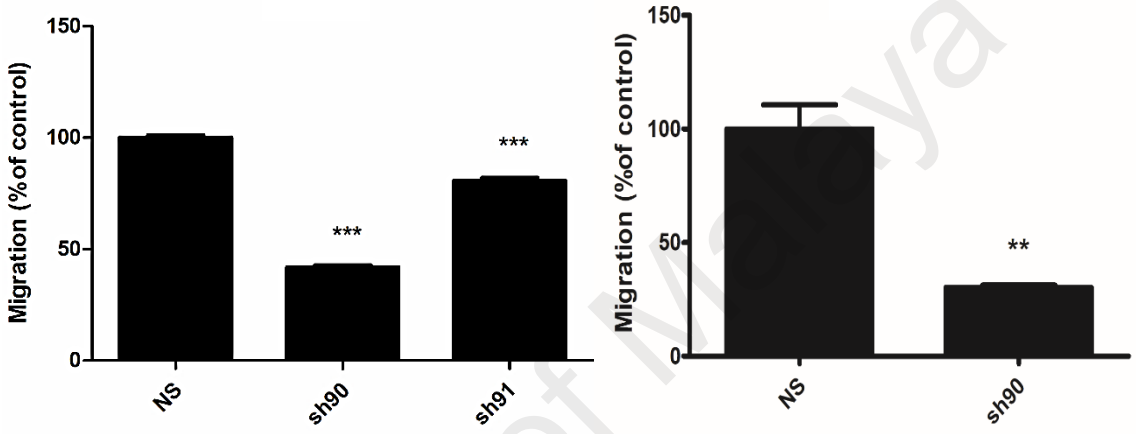



**Figure 4.14: Knockdown of LPAR3 expression inhibited OSCC cell migration.**
The effects of silencing the expression of *LPAR3* on OSCC cell migration were examined in fibronectin-coated transwell assays in the presence of 10µM LPA in 1%FBS in the lower chamber. There were significant attenuations of H357 cell migration in cell transfected with sh90 and sh91 as compared to control (NS). BICR31 cell transfected with sh90 also resulted in a significant attenuation of cell migration compared to control (NS) (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Dunnett's test). Graphs are representative of three experiments.

### 4.2.10 LPA protected OSCC cell lines to the effects of low dose radiation.

LPA has been reported to reduce apoptosis of nontransformed intestinal crypt-derived epithelial cell lines after high dose radiotherapy (>10Gy) (Deng et al., 2002). Another *in vitro* study showed that radiotherapy was more likely to induce apoptosis in LPAR2-deficient cells (Kiss et al., 2013). This study investigated the protective effect of LPA in OSCC cell lines when exposed to radiation. Clonogenic assays were used to optimise the radiation doses for for two selected OSCC cell lines (H357 and BICR31). A dose-dependent effect of irradiation on cell survival was observed (Figure 4.15). Cell surival was less than 50% when the cells were exposed to doses in the range of 2-8 Gy. Therefore, only doses of 1 and 2 Gy were used in subsequent experiments.

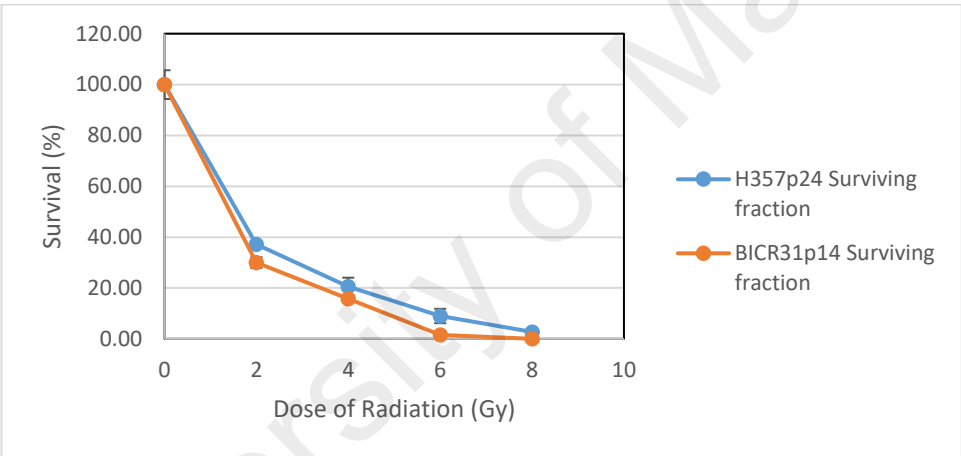



**Figure 4.15: Percentage of Survival fractions (SF) of OSCC cell lines after being irradiated at low (one or two Gy) until high dose (8 Gy).**
Data represents the results from three independent experiments.

The survival fractions of H357 cells irradiated at one and two Gy decreased significantly when compared to control (Figure 4.16). Pre-treatment with LPA for one hour or overnight, increased the survival fractions significantly for one ($p$<0.05) and two Gy ($p$<0.01). Similar results were observed in BICR31 (Figure 4.17) but only when exposed to two Gy irradiation. This result shows the potential of LPA in rescuing the irradiated OSCC cell lines.

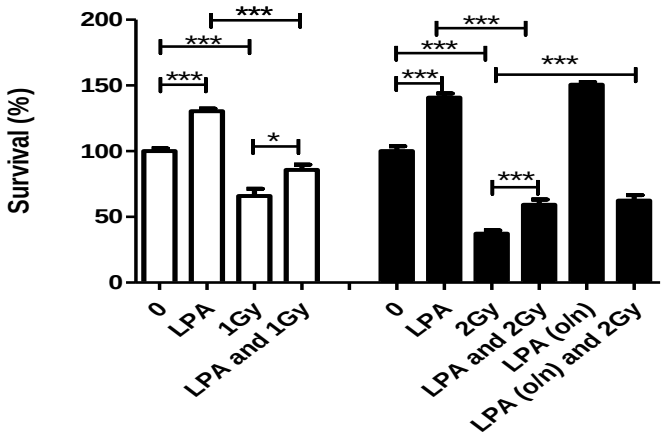



**Figure 4.16: LPA protects H357 (OSCC cell line) against one and two irradiation doses (one and two Gy).**

Percentage of cell survival fractions increased significantly after irradiation at one Gy (white bars) and two Gy (black bars). The cells were pre-treated with LPA (10µM) for one hour before irradiation. Overnight pre-treatment of LPA resulted in similar results as one hour of LPA pre-treatment. Data represents the results from three independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates. (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

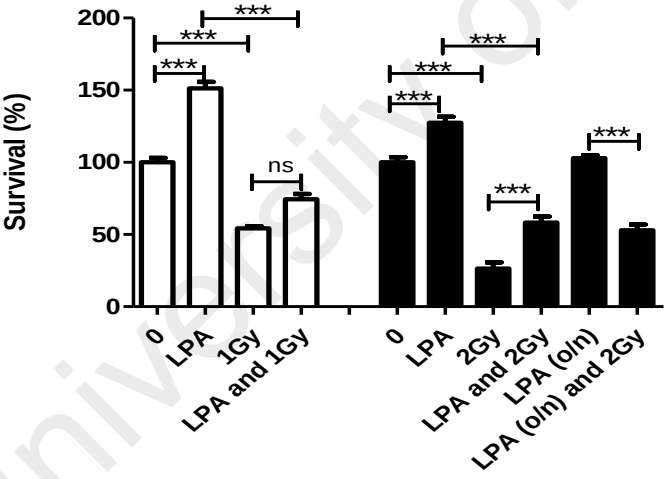



**Figure 4.17: LPA protects BICR31 (OSCC cell line) against low irradiation dose (two Gy).**

Percentage of cell survival fractions increased significantly after irradiation at two Gy (black bars). The cells were pre-treated with LPA (10µM) for one hour before irradiation. Overnight pre-treatment of LPA resulted in similar results as one hour of LPA pre-treatment. Data represents the results from three independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates. (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

### 4.2.11 The LPAR1/3 inhibitor Ki16425 decreased cell survival in H357 but not in BICR31 in low doses of irradiation treatment.

To investigate the role of LPAR1/3, experiments were performed in the presence or absence of the LPAR1/3 inhibitor, Ki16425. Two OSCC cell lines (H357 and BICR31) were pre-treated with Ki16425 for one hour before adding LPA for another hour. The cells were exposed to low doses of radiation and clonogenic survival measured after two weeks. Pre-treatment with Ki16425 significantly decreased the survival fraction of H357 when exposed to one Gy irradiation (Figure 4.18) but not a two Gy. No significant difference was observed in BICR31 cells (Figure 4.19). These observations suggest a role for LPAR1/3 in regulating the response of specific OSCC cells to radiation.

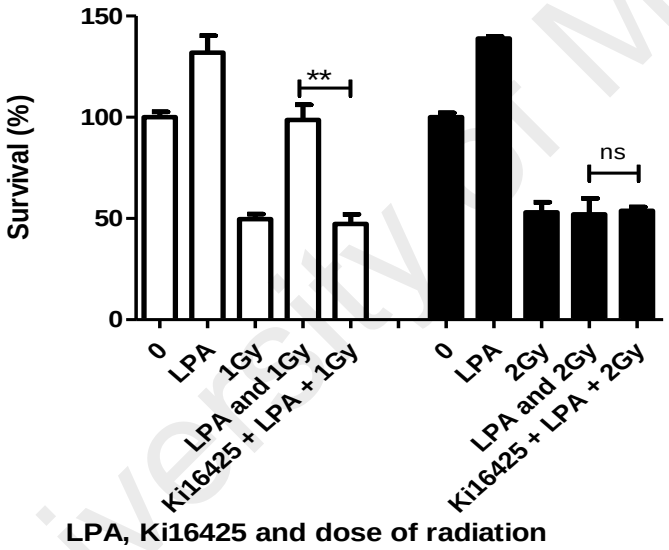



**Figure 4.18: LPAR1/3 inhibitor Ki16425 reverses the effect of LPA on cell survivability of H357 (OSCC cell line) following irradiation (one Gy).**
Percentage of cell survival fraction decreased significantly after pre-treatment with Ki16426 for one hour at one Gy (white bars). The cells were pre-treated with LPA (10µM) and Ki16425 for another hour before irradiation. There was no protection at irradiation of two Gy (black bars). Data represents the results from at least three independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates. (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

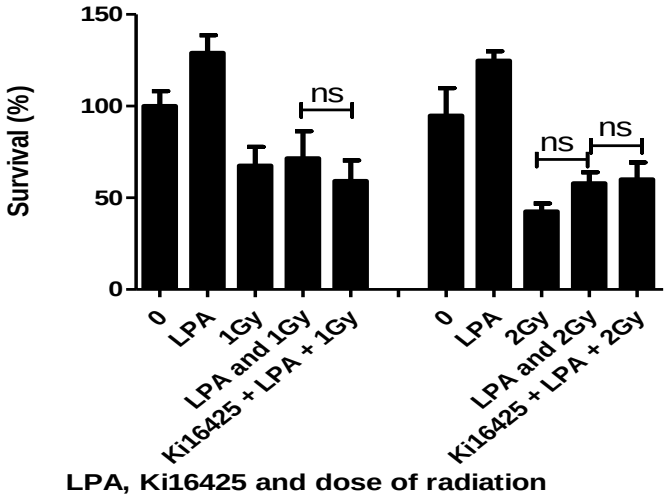



**Figure 4.19: LPAR1/3 inhibitor Ki16425 does not protect BICR31 (OSCC cell line) following irradiation (one or two Gy).**
Percentage of cell survival fraction was decreased but it was insignificant after pre-treatment with Ki16426 for one hour at one Gy. Although LPA seemed to protect against irradiation at two Gy, the increased of cell survival was insignificant. There was also no protection by Ki16425 at irradiation of two Gy. Data represents the results from at least three independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates. (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test)**.**

## 4.3 Summary

This study examined the expression of genes related to LPA synthesis and signalling in OSCC and subsequently investigated the functional effect of LPA in migration, invasion and radiation resistance of OSCC cells *in vitro*. The mRNA levels of *ENPP2* and *LPAR3* were examined in 52 OSCC tissue samples and 5 control tissues. Overall, *LPAR3* but not *ENPP2* was up-regulated in OSCC tissues as compared to normal oral mucosa ($p<0.05$). However, a subset of these tumour cases over-expressed *LPAR3* or ENPP2. Spearman correlation test showed a low and positive correlation between the expressions of these two genes. Pearson's Chi-square test showed that betel nut chewing is associated with high expression of *LPAR3* and *ENPP2*. *ENPP2* but not *LPAR3* showed a significant association with cancer stage.

Exogenous addition of LPA significantly increased the migration of H357 and H376 in transwell migration assays ($p<0.001$). OSCC cell migration was inhibited by the

LPAR1/3 antagonist, Ki16425. LPA-induced invasion of H357 was also significantly inhibited by Ki16425. Knockdown of LPAR3 in two OSCC cell lines attenuated cell migration. These results demonstrate that LPA stimulates cell migration and invasion through the activation of LPAR3.

Another downstream effect of the LPA/LPAR axis that is clinically important is resistance to radiotherapy. In the present study, LPA increased the survival of OSCC cell lines exposed to low irradiation doses (1/2 Gy). Pretreatment with LPAR1/3 inhibitor, Ki16425, was found to decrease cell survival in one of two cell lines that were treated with one Gy of radiation in the presence of LPA. This suggested that LPAR1/3 may be involved in the survival of the cells and it is likely to be tumour cell specific.

In summary, *LPAR3* is overexpressed in a sub-set of OSCCs, possibly leading to activation of downstream signalling pathways that result in increased migration, invasion and resistance to radiation.

# CHAPTER 5:

# CROSSTALK BETWEEN LPA, EGFR AND COX-2 AND ITS FUNCTIONAL SIGNIFICANCE IN ORAL SQUAMOUS CELL CARCINOMA.

## 5.1 Introduction

LPA receptors belong to a large group of receptors called GPCRs. Stimulation of GPCRs can lead to transactivation of the EGFR causing subsequent activation of intracellular signalling cascades (Daub et al., 1996). Functional crosstalk between GPCRs and EGFR has been shown to contribute to the progression of tumours, such as colon (Cheng et al., 2003), lung (Luppi et al., 2007), breast (Filardo et al., 2000), ovarian (Miyamoto et al., 2004), prostate (Kue et al., 2002) and in HNSCC (S. M. Thomas et al., 2006). In a variety of HNSCC cell lines, for example, treatment with GPCR agonists such as LPA, bradykinin, thrombin and carbacol resulted in rapid tyrosine phosphorylation of EGFR (Gschwind et al., 2002).

Another important downstream pathway that has been shown to be activated in cancer is the COX-2 pathway. COX-2 is an enzyme that functions in protein metabolism by increasing prostaglandin synthesis and plays a role in tumorigenesis by converting arachidonic acid to prostaglandin $H_2$ (PGH-2); PGH-2 is then converted to prostaglandins E2 (PGE-2) which can stimulate cancer progression (Menter et al., 2010).

LPA has been shown to induce COX-2 expression in human colon cancer cells (Shida et al., 2005a). LPA effected the transcription activation and post-transcriptional enhancement of COX-2 mRNA stability in ovarian cancer cells (Oyesanya et al., 2008). Overexpression of COX-2 has been shown in OSCC patients with significant association of lymph node involvement, histological grade, local recurrence of tumour and patient survival (Byatnal et al., 2015). Interestingly, the bioactive lipid, S1P, has been shown in our lab to up-regulate COX-2 in OSCC cell lines (Patmanathan, 2016), but whether LPA can also induce COX-2 expression in OSCC cell lines has not been investigated. In this

study, the potential crosstalk between LPA/LPAR3 and both EGFR and COX-2 was investigated.

## 5.2 Results.

### 5.2.1 EGFR and COX-2 expression in OSCC cell lines

The endogenous levels of total EGFR, phosphorylated EGFR (p-EGFR) and COX-2 were first examined in a panel of eight OSCC cell lines by Western blotting. The OSCC cell lines showed varied expression of the proteins (Figure 5.1). H357, H376 and H400 were selected for further study because they expressed low to moderate levels of p-EGFR and COX-2.

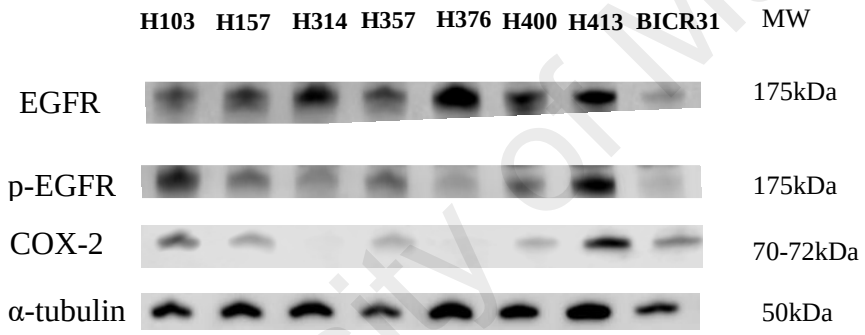



**Figure 5.1: Expression of total EGFR, phosphorylated EGFR (p-EGFR) and COX-2 in OSCC cell lines.**
Proteins were extracted from OSCC cell lines cultured in complete medium and subjected to Western blot analysis to determine the levels of total EGFR, phosphorylated EGFR (p-EGFR), COX-2 and α-tubulin (endogenous control).Data are representative of at least three independent experiments.

### 5.2.2 Effect of LPA on EGFR expression and phosphorylation of OSCC cell lines.

To begin to study potential crosstalk between LPA and EGFR in OSCC cells, the effect of LPA on EGFR phosphorylation was examined. For all three OSCC cell lines, (H357, H376 and H400) (Figure 5.2), basal phosphorylation of EGFR was evident in the absence of LPA (time zero). Although total EGFR appeared to be decreased up to eight-hour time point in H357 and H376 cells, there was no consistent induction of EGFR phosphorylation by LPA.

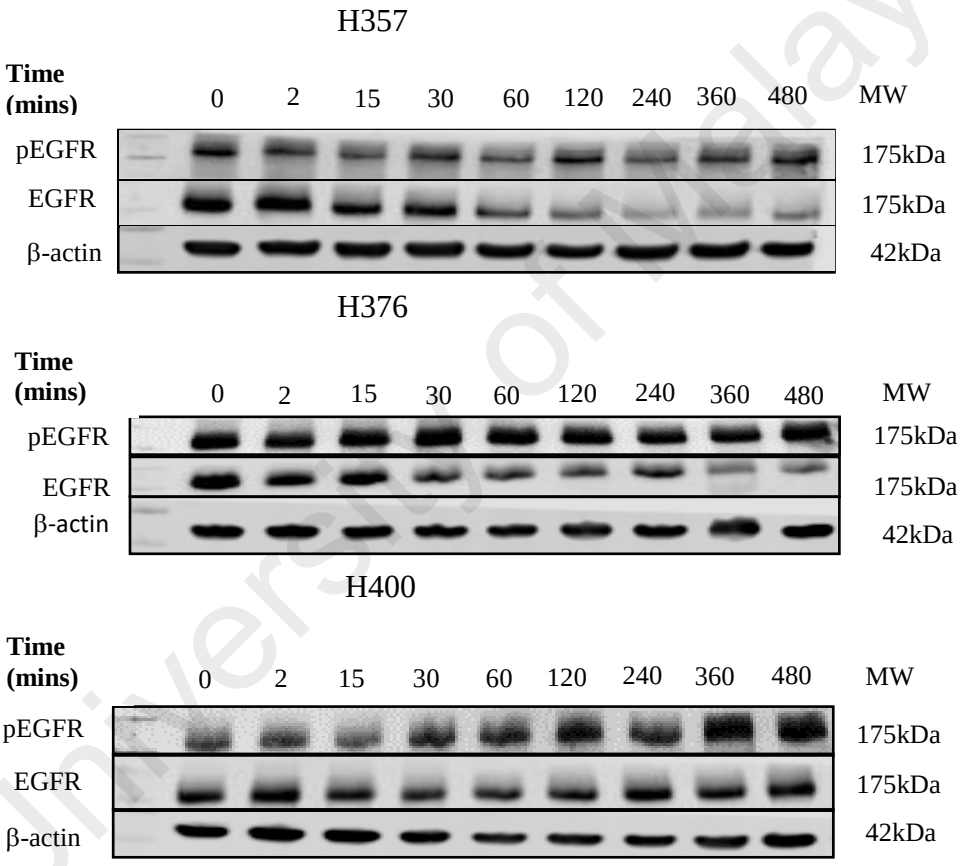



**Figure 5.2: Effect of LPA on EGFR phosphorylation**.
H357, H376 and H400 were serum starved for 24 hours. Cells were treated with 10μM LPA in serum-free media and cell lysates were collected according to the indicated time (minutes). Protein lysates (50ug/sample) were prepared and subjected to SDS-PAGE and immunoblotting to assess phosphorylation of EGFR. Phosphorylation of EGFR was not clear for all time points. H376 and H400 have high baseline levels of phosphorylated EGFR and remained phosphorylated throughout the treatments. Data are representative of at least three independent experiments.

### 5.2.3 Effect of LPA on COX-2 expression in OSCC cell lines.

H357, H376 and H400 cells were first serum-starved for 24 hours before treatment with 10µM LPA in serum-free media. COX-2 protein levels were assessed by immunoblotting. Treatment of all cell lines with LPA resulted in a clear increase in COX-2 protein levels after two hours of treatment, which peaked after 4-6 hours (Figure 5.3).

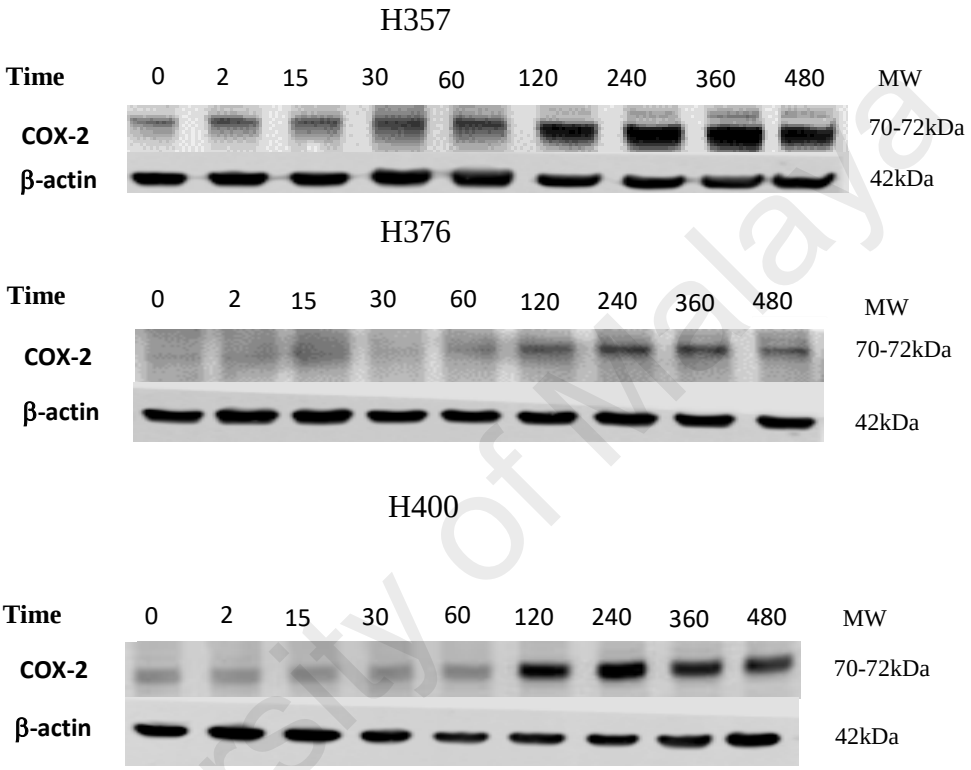


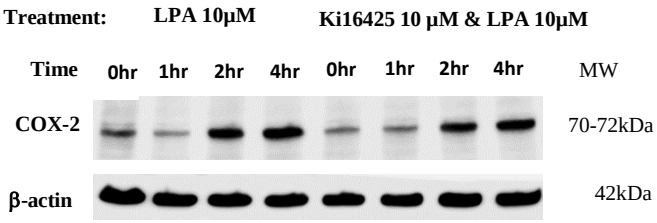

**Figure 5.3: LPA induces COX-2 expression in OSCC cell lines.**
H357, H376 and H400 were serum starved for 24 hours. Cell were treated with 10µM LPA in serum-free media according to the indicated time (minutes). Protein lysates were prepared and subjected to immunoblotting to assess COX-2 and β-actin protein expression. All three cell lines showed increased in COX-2 protein expression after 2 hours of LPA treatment. Data are representative of at least three independent experiments.

### 5.2.4 Role of LPAR3 in the regulation of COX-2 expression in OSCC cell lines.

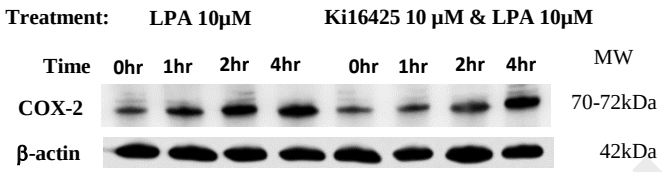
To begin to assess the role of LPAR3 in regulating COX-2 expression, OSCC cells were pre-treated with the LPAR1/3 inhibitor, Ki16425, for 60 minutes prior to stimulation with LPA. BICR was included in these experiments as BICR31 cells stably expressing shRNAs targeting *LPAR3* were generated previously, as described in Chapter 4. In all cell lines, Ki16425 attenuated the induction of Cox-2 expression by LPA (Figure 5.4), suggesting the involvement of LPAR1 or LPAR3 in mediating this effect.

To specifically investigate the role of LPAR3 in mediating LPA-induced COX-2 expression, H357 and BICR31 cells that were previously engineered to stably express *LPAR3* shRNAs (Section 4.2.9) were utilised. These transfected cells were treated with LPA and COX-2 was detected by Western blotting after 1-2 hours. LPA caused a transient increase of COX-2 after 1 hour in H357 cells, but decreased COX-2 levels were apparent in both cell lines after two hours of LPA treatment (Figure 5.5). These data demonstrate the involvement of LPAR3 in regulating the levels of COX-2 in OSCC cell lines. However data on BICR31 cell line in Chapter 4 suggests regulators of COX-2 other than LPAR3 exist. This was based on the fact that inhibition of LPAR3 in BICR31 did not overcome the radio protective effect of LPA in this cell line.

**(A) H357**




**(B) BICR31**




**(C) H400**

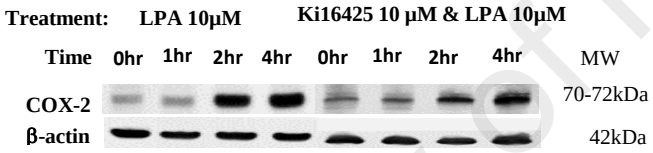
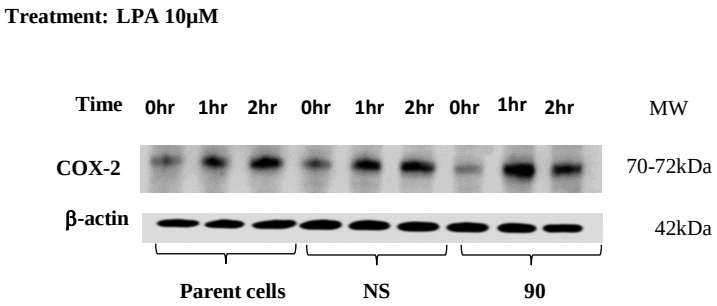



**Figure 5.4: Effect of LPAR1/3 inhibition by Ki16425 in COX-2 expression in OSCC cells**.
In all cell lines (H357, H400 and BICR31), COX-2 was clearly expressed after two and four hours of LPA treatment. Pre-treatment with Ki16425 attenuated COX-2 levels at two and four hours. Data represents the results from at least three independent experiments.

(A) H357




(C) BICR31

(D)

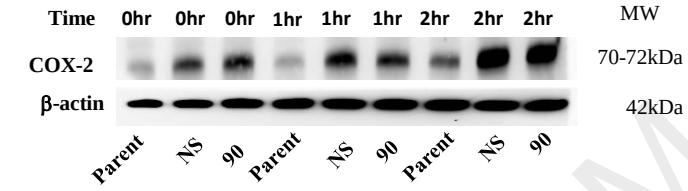



**Figure 5.5: Knockdown of LPAR3 expression attenuated LPA-induced COX-2 protein level.**

The effects of silencing the expression of *LPAR3* on LPA-treated OSCC cell lines. In both cell lines, COX-2 levels increased moderately until two-hour treatment time point for both parent and scrambled control groups. COX-2 level decreased after two hours when compared to scrambled control. This result shows the involvement of LPAR3 in regulation of COX-2 levels. Data represents the results from at least two independent experiments.

### 5.2.5 Effect of a selective COX-2 inhibitor NS 398 on LPA-induced OSCC cell migration.

As LPA was shown to increase COX-2 protein levels and enhance cell migration in OSCC cell lines, it was hypothesized that COX-2 might mediate some of the pro-migratory effects of LPA. In this study, 10 µM NS 398 was used to inhibit COX-2 activity, as this concentration showed no toxicity cytotoxic towards OSCC cells (data not shown) and has been used in previous studies to inhibit COX-2 (Morita et al., 2012; Shida et al., 2005a).

LPA-induced migration of H357 and H376 cells was significantly attenuated following COX-2 inhibition with NS 398 in transwell migration assays (Figure 5.6).

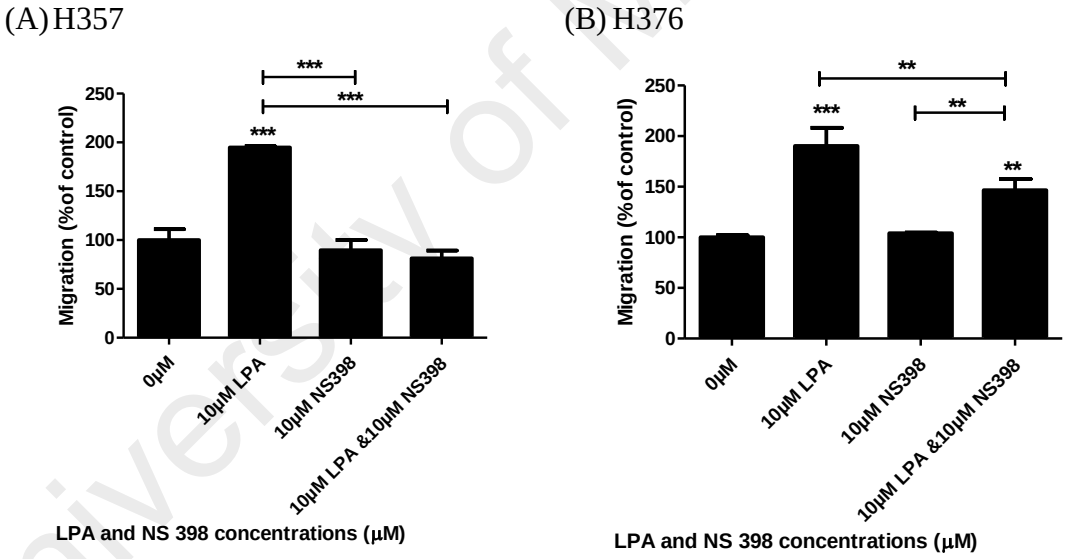



**Figure 5.6: COX-2 inhibitor NS 398 reduces LPA-induced OSCC cell migration.** In both H357 and H376, NS 398 has significantly reduced cell migrations. H357 and H376 were cultured in media with reduced serum (1%FBS) for 24 hours. The cell lines were then treated with mitomycin C (10ug/mL). Treatment with vehicle control (50% Ethanol), 10µM LPA, 10µM NS398 and a combination of both LPA and 10µM NS398 were carry out for 18hours in transwell migration assay. Cells were washed with PBS and stained with 0.1% crystal violet in methanol for 2.5hours. Data represents the results from at least three independent experiments. Bars, mean; error bars, standard deviation (SD). (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

### 5.2.6 Effect of a selective COX-2 inhibitor NS 398 on OSCC cell survival following exposure to low dose radiation.

To investigate the involvement of COX-2 in mediating the effects of LPA on OSCC cell survival following radiation, cells were pre-treated with NS398 for one hour prior to incubating the cells in LPA for one hour and irradiation treatment. The cells were exposed to one Gy of irradiation and clonogenic survival measured after two weeks. In H357, NS398 was able to decrease the survival of the LPA pre-treated and irradiated cell lines ($p<0.01$). BICR31 showed similar trend but the decreased of the cell survival was not significant. These observations demonstrate that COX-2 is involved in the survival of OSCC cells induced by LPA. However this effect may be cell specific.

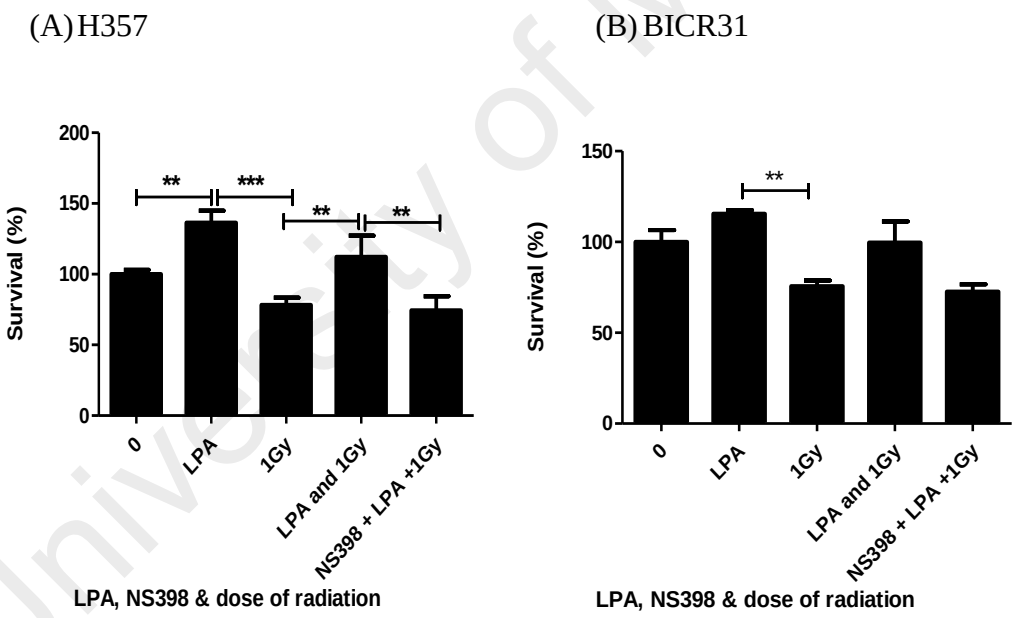



**Figure 5.7: COX-2 inhibitor NS398 reduces H357 cell survival when exposed to low irradiation dose (one Gy).**
Percentage of cell survival fraction of H357 decreased significantly after pre-treatment with NS398 for one hour at one Gy. The cells were pre-treated with LPA (10µM) and NS398 for another hour before irradiation. Results for BICR31 showed similar trend but the difference between groups treated with NS398 was not significantly different compared to non-treated group. Data represents the results from at least three independent experiments. Bars represent means of triplicates; error bars represent SD of triplicates. (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ in Tukey's test).

## 5.3 Summary

This study examined the effect of LPA on EGFR phosphorylation and COX-2 expression in OSCC cells. The functional significance of LPA-induced up-regulation of COX-2 was also investigated. Treatment of OSCC cell lines with LPA (10μM) did not show any increase in EGFR phosphorylation. This may be due to high basal phosphorylation of EGFR in these cell lines. If there are subtle differences between total and non-phosphorylated EGFR, other technique such as the use of mass-spectrometry should be used in the future in order to detect any differences. Treatment of OSCC cells with LPA (10μM) induced a transient increase in COX-2 protein levels and this effect was abrogated when the cells were pre-treated with the LPAR1/3 inhibitor (Ki16425). Further investigations were carried out using cells stably expressing shRNAs to knockdown LPAR3. In cells with reduced LPAR3 protein, there was a transient increase of COX-2 after one hour but levels decreased back to baseline after two hours. This result supports the involvement of LPAR3 in regulating the levels of COX-2 in OSCC cell lines. In order to investigate the functional significance of LPA-induced COX-2 expression, the effects of a COX-2 inhibitor, NS398 was examined. LPA-induced migration of H357 and H376 was significantly attenuated following COX-2 inhibition with NS 398 in transwell migration assays. NS398 was also able to reduce cell survival of one of two OSCC cell lines when cells were pre-treated with LPA and exposed to one Gy of irradiation. In summary, the results of this study demonstrate that COX-2 mediates some of the effects of LPA-LPAR3 promotion of OSCC cell migration and radioresistance.

# CHAPTER 6:

# THE POTENTIAL OF PLASMA LPA AS A BIOMARKER IN ORAL SQUAMOUS CELL CARCINOMA.

## 6.1 Introduction

LPA influences many normal physiological and biological processes. Increasing evidence points to a role for LPA in pathophysiological processes for example, wound healing, atherosclerosis and cancer. The highest LPA levels are found in serum (Baker et al., 2000), although the lipid can also be found in plasma (Scherer et al., 2009; Zhang et al., 2015), saliva (Bathena et al., 2011; Sugiura et al., 2002), ascites fluids (Xiao et al., 2001), pleural effusions (Bai et al., 2014) and follicular fluids (Tokumura et al., 1999; Yamamoto et al., 2016). LPA has been proposed as a potential biomarker for ovarian cancer and other gynaecological malignancies because higher plasma levels have been observed in cancer patients compared to healthy controls (Cao et al., 2015; Xu et al., 1998; Zhang et al., 2015). LPA levels were also reported to be elevated in the serum of multiple myeloma patients (Sasagawa, Oka, et al., 1999). However, to the best of my knowledge, no study has been carried out to detect LPA in OSCC patients.

Previous work in our laboratory has detected LPA in tissue culture supernatants from OSCC cell lines using Enzyme-Linked Immunosorbent Assay (ELISA; Johnson and Paterson, unpublished data). Furthermore, the results of Chapter 4 demonstrated that ATX was highly expressed in a small subset of OSCC patients. Taken together, these data suggest that LPA might have the potential to be used as a biomarker for OSCC. Therefore, the aim of the present study was to measure LPA levels in plasma samples from OSCC patients and normal controls and to correlate the results with clinicopathological parameters of the patients.

## 6.2 Results

### 6.2.1 General features of the LCMS/MS analysis

A high-performance liquid chromatography mass spectrometric method for the estimation of LPA levels in plasma samples of OSCC was developed in this study. Validation of the method was performed according to the USFDA guidelines (U.S. Department of Health and Human Services, 2001). The methodology is described in detail in Section 3.4. Chromatograms of the individual LPAs with their retention times with a total run time of 5 mins are shown in Figure 6.1.

All LPA species were analysed in negative ion mode. From the LCMS/MS analyses, the mass spectra of the LPA species showed a deprotonated molecular ion $[M-H]^-$ at m/z 409.2. 16:0 LPA is shown as an example (Figure 6.2A) and high energy generated from cell collision, fragmented this 16:0 LPA molecular ion into several fragments. The major fragment observed for 16:0 LPA was at m/z 153 and this fragment ion was selected for subsequent monitoring in the third quadrupole of the mass spectrometer. Figures 6.2B-6.2F show representative peaks for all LPA species analysed.

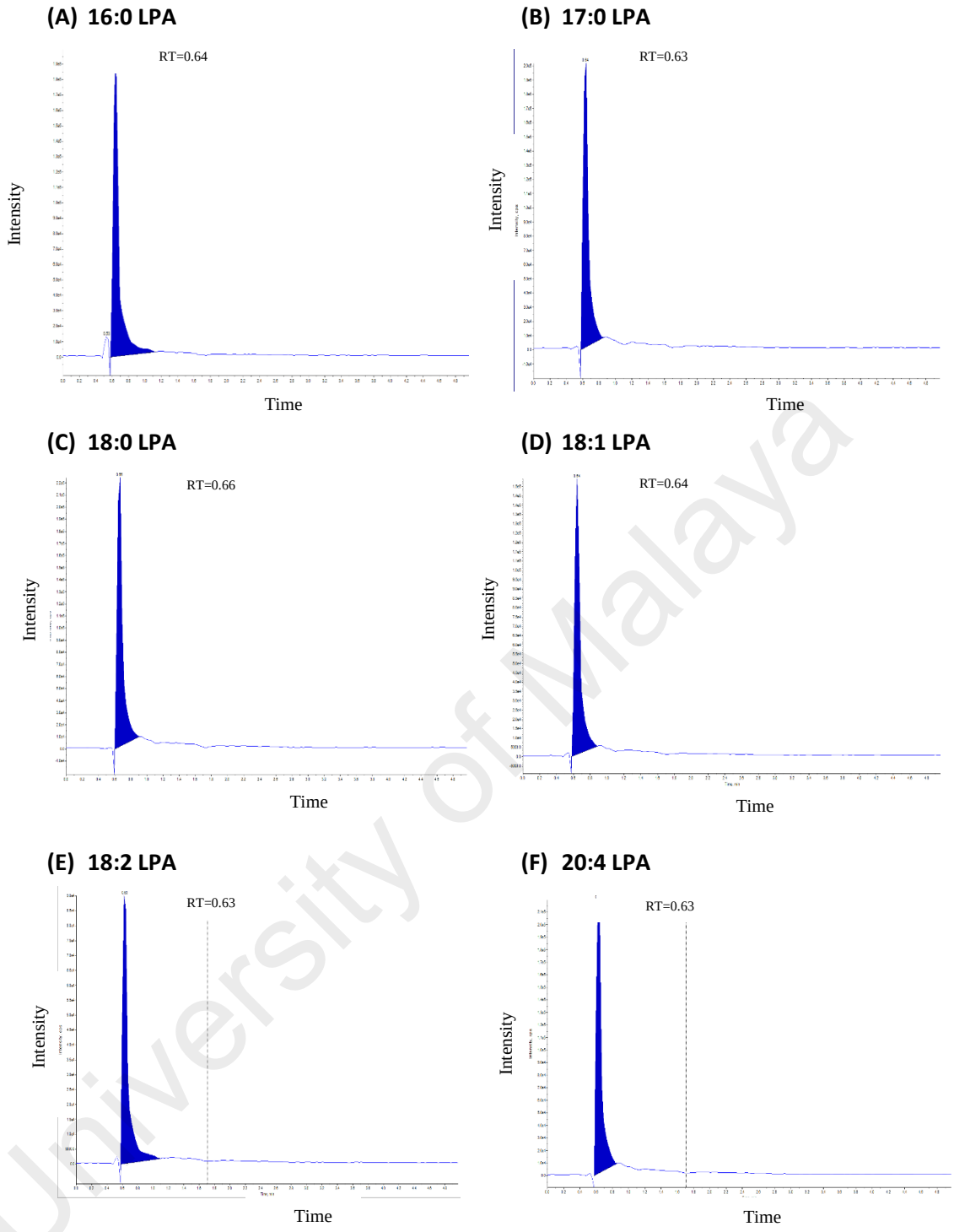





**Figure 6.1: Representative MRM chromatograms of individual LPAs in blank serum at 1500ppb of the standard curve.**

Individual retention times for each LPA are as follows: 16:0 LPA-0.642min, 17:0 LPA-0.636min, 18:0 LPA-0.661min, 18:1 LPA-0.644min, 18:2 LPA-0.631 and 20:4 LPA-0.630min. 17:0 LPA was the internal standard incorporated in all standard mixtures and samples for normalization of all runs. The overall run time for chromatography was 5 mins.

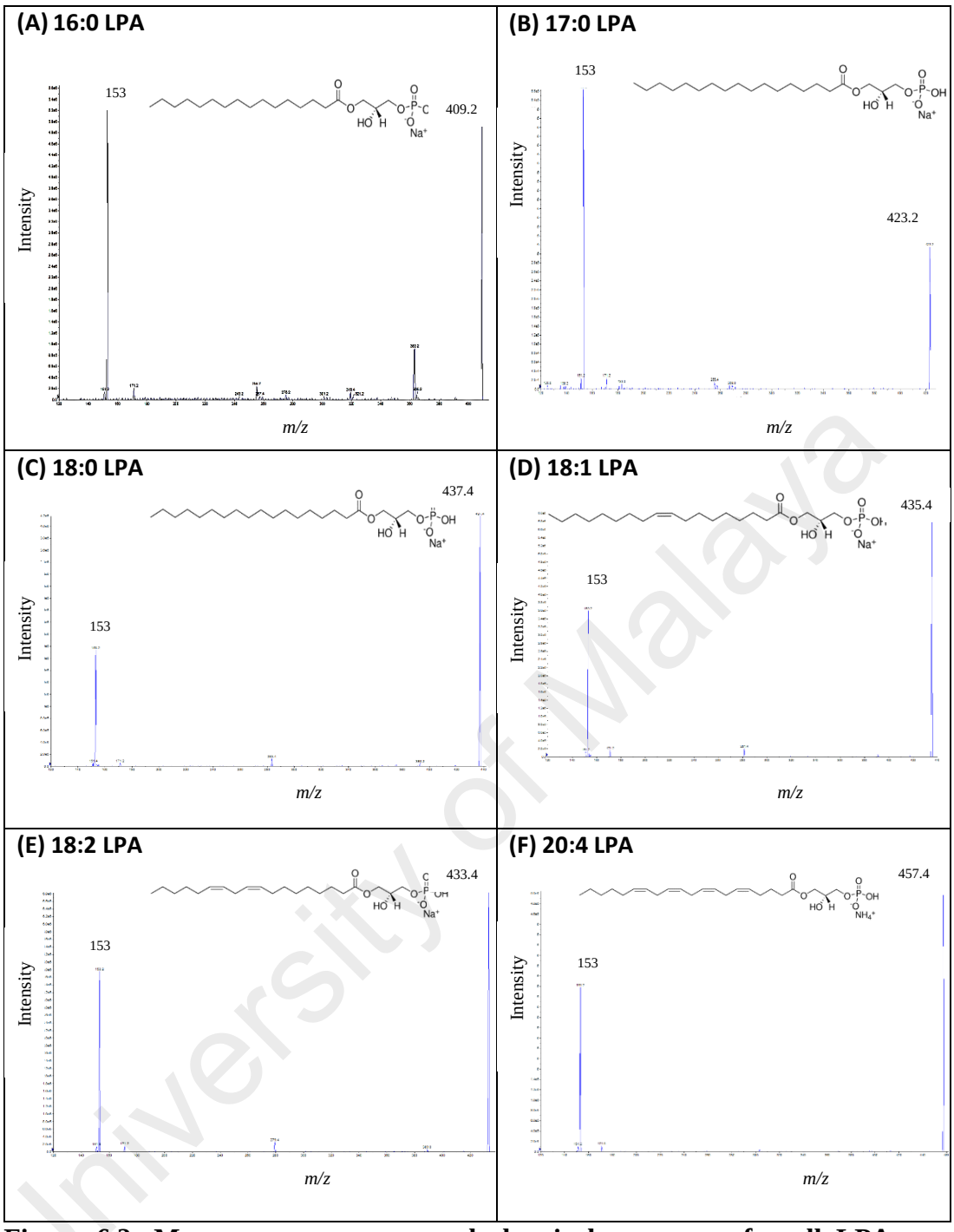



**Figure 6.2: Mass spectrum scans and chemical structures for all LPA species examined.**

The MRM transitions used to detect LPA were the mass to charge ration (*m/z*) for the molecular anion $M^-$ and their daughter ion *m/z* 153 (16:0 LPA (*m/z* 409→153) , 17:0 LPA (*m/z* 423→153), 18:0 LPA (*m/z* 437→153), 18:1 LPA(*m/z* 435→153), 18:2 LPA (*m/z* 433→153) and 20:4 LPA (*m/z* 457→153).

### 6.2.2 Clinicopathological characteristics of OSCC.

120 plasma samples from OSCC patients (n=80) and control healthy individuals (n=40) were obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCCTBS) managed by the Oral Cancer Research and Coordinating Centre (OCRCC), University of Malaya. Plasma from control subjects was collected concurrently from healthy individuals with no history of cancer. Information regarding the gender, age, risk factors and cancer stage are shown in Table 6.1. All participants provided written informed consent. Ethical approval for this study was obtained from the Medical Ethics Committee, Faculty of Malaya (DF OB1403/0009L).

**Table 6.1: Clinicopathological characteristics of OSCC and normal plasma samples.**

| **Characteristics** | | **Normal (n=40) (%)** | **Tumour (n=80) (%)** |
|---|---|---|---|
| Gender | Male | 19 (47.50) | 29 (36.25) |
| | Female | 21 (52.50) | 51 (63.75) |
| Age (years) | ≤ 50 | 27 (67.50) | 22 (27.50) |
| | ≥ 50 | 13 (32.50) | 58 (72.5) |
| Smoking status | Smokers | 3 (7.50) | 27 (33.75) |
| | Non-smokers | 37 (92.50) | 53 (66.25) |
| Betel chewing status | Chewers | 3 (7.50) | 37 (46.25) |
| | Non- chewers | 37 (92.50) | 43 (53.75) |
| Drinking | Drinkers | 8 (20.00) | 20 (25.00) |
| | Non-drinkers | 32 (80.00) | 60 (75.00) |
| Stage | Early (I&II) | N/A | 29 (36.25) |
| | Advanced (III&IV) | N/A | 51 (63.75) |

N/A- not applicable

### 6.2.3 LPA levels in plasma samples of OSCC patients and normal subjects.

The aim of this study was to measure the plasma LPA levels in OSCC patients. Table 6.2 summarizes the concentration of LPA species obtained from healthy control subjects and OSCC patients in the early (stage I and II) or advance stages (stage III and IV) of the disease. 16:0 LPA, 18:0 LPA, 18:1 LPA and 18:2 LPA were major LPA species found in these plasma samples, whereas 20:4 LPA was only a minor component. The order of abundance of the LPA species found in normal plasma samples is as follows: LPA: 18:2>16:0>18:0>18:1>20:4 which is similar to another study by Shan et. al. where both 18:2 LPA and 16:0 LPA were also found to be in the highest concentration following the same order (Shan et al., 2008). Baker *et. al,* 2002 also observed a similar pattern where 18:2 LPA was the highest concentrated LPA species, followed by 20:4 LPA, 16:0 LPA,18:1 LPA and 18:0 LPA in normal samples (Baker et al., 2002). The profiles of LPA species detected in both early and advance cancer groups were the same which is LPA 16:0>18:0>18:2>18:1>20:4>14:0. The profiles for both cancer groups were different than the profile of normal plasma samples.

**Table 6.2: LPA levels in plasma of normal subjects and OSCC patients with early or advance cancer. Early – stage I and II and advance – stage III and IV.**

| OSCC | Plasma samples | N (number of samples) | Mean(μM) ± SEM |
|---|---|---|---|
| LPA 16.0 | NORMAL | 40.00 | 37.92 ± 2.45 |
| | EARLY CANCER (stage I and II) | 40.00 | 36.66 ± 2.69 |
| | ADVANCE CANCER (stage III and IV) | 40.00 | 37.24 ± 2.33 |
| LPA 18.0 | NORMAL | 40.00 | 37.20 ± 2.23 |
| | EARLY CANCER (stage I and II) | 40.00 | 31.85 ± 2.39 |
| | ADVANCE CANCER (stage III and IV) | 40.00 | 31.46 ± 1.95 |
| LPA 18.1 | NORMAL | 40.00 | 24.59 ± 1.89 |
| | EARLY CANCER (stage I and II) | 40.00 | 18.05 ± 1.59 |
| | ADVANCE CANCER (stage III and IV) | 40.00 | 17.61 ± 1.14 |
| LPA 18.2 | NORMAL | 40.00 | 39.79 ± 2.48 |
| | EARLY CANCER (stage I and II) | 40.00 | 20.96 ± 2.24 |
| | ADVANCE CANCER (stage III and IV) | 40.00 | 21.51 ± 1.68 |
| LPA 20.4 | NORMAL | 40.00 | 7.26 ± 0.49 |
| | EARLY CANCER (stage I and II) | 40.00 | 5.74 ± 0.53 |
| | ADVANCE CANCER (stage III and IV) | 40.00 | 6.13 ± 0.41 |
| TOTAL | NORMAL | | 146.77 ± 7.98 |
| | EARLY CANCER (stage I and II) | | 112.98 ± 8.79 |
| | ADVANCE CANCER (stage III and IV) | | 113.96 ± 6.54 |

### 6.2.4 LPA profiling of OSCC patients and normal controls using univariate (box-plot).

To examine the variation in individual LPA levels between normal and OSCC samples, univariate analysis using box-plots were constructed using data from quantitative MS analyses. This was then used to illustrate the differences of LPA levels between normal, early and advanced OSCC groups as shown in Figure 6.3. There were no significant differences in LPA levels between the normal, early and advanced groups in 16:0 LPA and 18:0 LPA (Figures 6.3A and 6.3B) when tested using the Kruskal-Wallis test ($p < 0.05$). By contrast, 18:1 LPA, 18:2 LPA and 20:4 LPA levels (Figures 6.3C, 6.3D and 6.3E) were significantly different between the three groups (normal, early and

advanced). To further identify which of the group differed from each other, post hoc tests were conducted between two groups (Table 6.3). In 18:1 LPA and 18:2 LPA, significant differences were detected in early-normal and advanced-normal groups whereas only early-normal were significantly different in 20:4 LPA. Small dots in the boxplots are data with extreme values in the data series and were not omitted from the analysis.

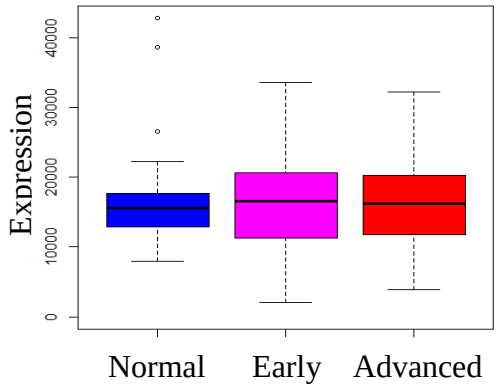
In summary, results from the boxplots showed that there were significant differences in between the three sample groups (normal, early and advanced) in three LPA species (18:1, 18:2 and 20:4. And within each LPA species, there were significant differences in early-normal and advanced-normal pairs in 18:1 LPA and 18:2 LPA. In 20:4, early-normal pair is also significantly different.

**Table 6.3: Kruskal-Wallis post-hoc test in pairing of two groups in 18:1 LPA, 18:2 LPA and 20:4 LPA.**

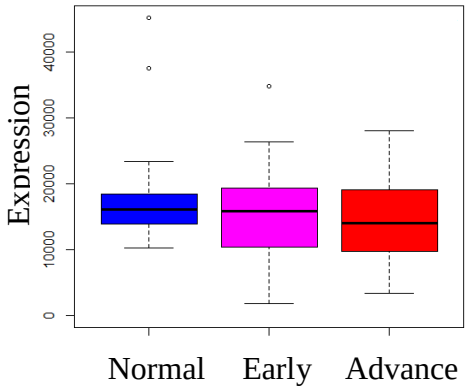
| **Pairwise comparison in between groups** | **18:1 LPA** | **18:2 LPA** | **20:4 LPA** |
|---|---|---|---|
| Early-advanced | 1.0 | 1.0 | 0.927 |
| Early-normal | **0.002*** | **<0.05*** | **0.027*** |
| Advanced-normal | **0.002*** | **<0.05*** | 0.267 |

***** Acceptable significance levels is when $p<0.05$.

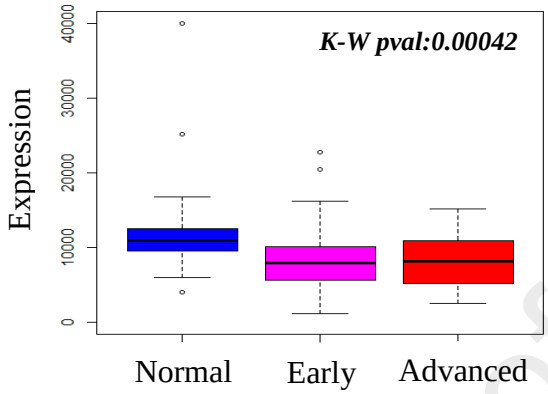
(A) 16:0 LPA



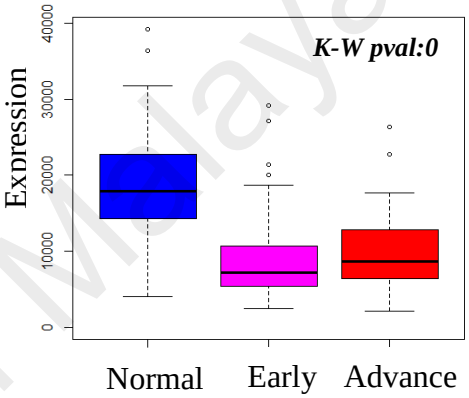

(B) 18:0 LPA




(C) 18:1 LPA




(D) 18:2 LPA




(E) 20:4 LPA

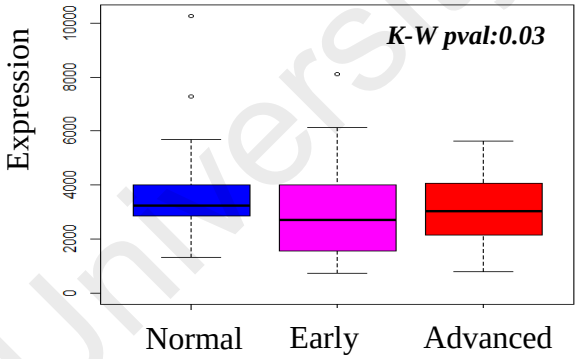



**Figure 6.3: Box plots (A-E) of the distribution of values for all five LPA species detected in the plasma samples of normal, early stage and advanced stage of oral squamous carcinoma groups**.

LPA levels were significant different among the three groups for 18:1 LPA, 18:2 LPA and 20:4 LPA (Figures 6C, 6D and 6E). K.W. represents Kruskal-Wallis test with $p < 0.05$.

### 6.2.5 LPA profiling of OSCC patients and normal controls using bivariate analysis (scatterplots).

After analysing the patterns of individual LPA species, the next step was to compare between two LPA species. In order to study the relationship between two LPA species, bivariate analyses using scatterplots were utilised with all possible pairings of the measured five LPA levels. All scatterplot results are shown in two figures (Figure 6.4A-6.4F and Figure 6.4G-6.4J). Figures 6.5A-6.5D showed scatterplots of 16:0 LPA with 18:0 LPA, 160:0 LPA with 18:1 LPA, 16:0 LPA with 18:2 LPA and 160:0 LPA with 20:4 LPA. Figures 6.5E-6.5G are scatterplots of 18:0 LPA with 18:1 LPA, 18:0 LPA with 18:2 LPA and 18:0 LPA with 20:4 LPA. Figures 6.5H-6.5I are the pairs for 18:1 LPA with 18:2 LPA and 20:4 LPA. The last figure (Figure 6.5J) was for the pair between 18:2 LPA and 20:4 LPA. Correlation studies have been conducted using Pearson correlation tests.

All scatterplots showed strong, linear and positive correlations between the three groups (normal, early and advanced). Only these LPA pairs (16:0 LPA with 18:2 LPA, 18:0 LPA with 18:2 LPA and 18:1 LPA with 18:2 LPA (Figures 6.5C, 6.5F and 6.5H) showed a weak, linear and positive correlations between the LPA pairs. Importantly, in these three LPA pairs, data points for normal samples were grouped together allowing possible differentiation between normal and cancer groups. Clusters of normal samples separated the normal group away from early and advanced cancer groups but not between early and advanced cancer groups. Clusters of normal samples are caused by the high levels of 18:2 LPA and the patterns were detected when it is paired with 16:0 LPA, 18:0 LPA and 18:1 LPA.

In order to study the relationship between the LPA species, correlations between any two LPA levels were analysed using Pearson correlation test (Table 6.4). The results indicated positive correlations in all LPA pairs. All LPA species showed strong

correlations with other LPAs except with 18:2 LPA (r: 0.7-1.0; $p<0.05$). 18:2 LPA showed moderate correlations with other LPA species (r: 0.3-0.7; $p<0.5$). In summary, there are distinctive clustering patterns of the normal samples with high levels of 18:2 LPA as one of the pairs when analysed using scatterplots. All LPA pairs showed positive correlations using the Pearson correlation test.

**Table 6.4: Correlation between two LPA species.**
Pearson correlation test was performed using values for two LPA species in each sample. Table shows Pearson correlation coefficients (r) for the LPA species. Dark blue box, strong correlation (r: 0.7-1.0); light blue box, moderate correlation (r: 0.3-0.7); white box. No correlation. Only positive correlations were observed in all analysis. ***=$p<0.001$, **$p<0.01$ and *=$p<0.05$

| LPA species | 16:0 LPA | 18:0 LPA | 18:1 LPA | 18:2 LPA | 20:4 LPA |
|---|---|---|---|---|---|
| **16:0 LPA** | | 0.901** | 0.819** | 0.409** | 0.816** |
| **18:0 LPA** | 0.901** | | 0.833** | 0.467** | 0.768** |
| **18:1 LPA** | 0.819** | 0.833** | | 0.666** | 0.908** |
| **18:2 LPA** | 0.409** | 0.467** | 0.666** | | 0.628** |
| **20:4 LPA** | 0.816** | 0.768** | 0.908** | 0.628** | |

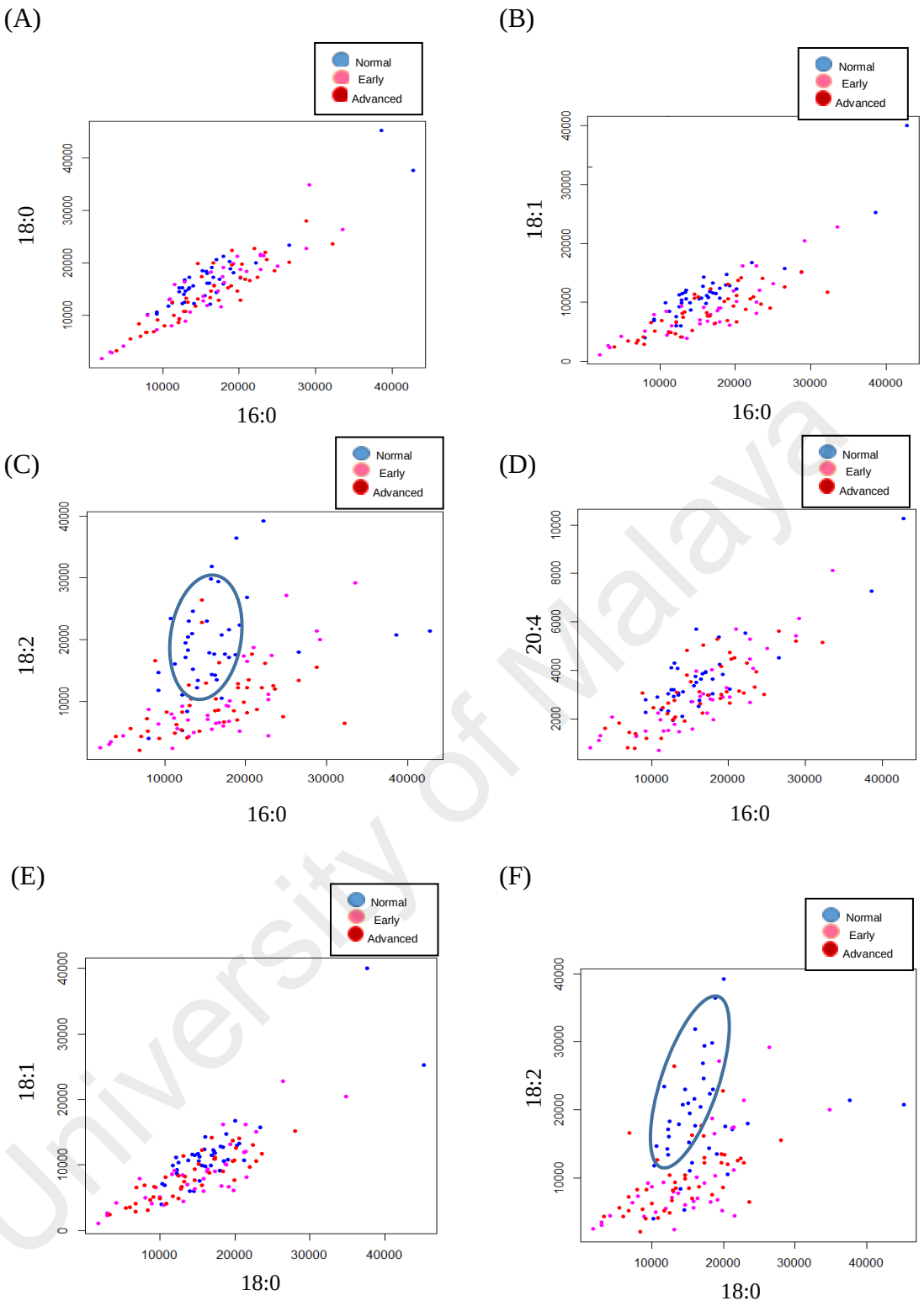





**Figure 6.4A-6.4F: Scatterplots of pairing of five LPA species in three OSCC groups (normal, early and advanced).**

Figure 6.4A-6.4D are figures for 16:0 LPA with 18:0, 18:1, 18:2 and 20:4 LPA. Figures 6.4E and 6.4F are for 18:0 with 18:1 and 18:2 LPA. Two pairs that contained 18:2 LPA showed separation of between normal groups with OSCC groups (blue ellipse diagram).

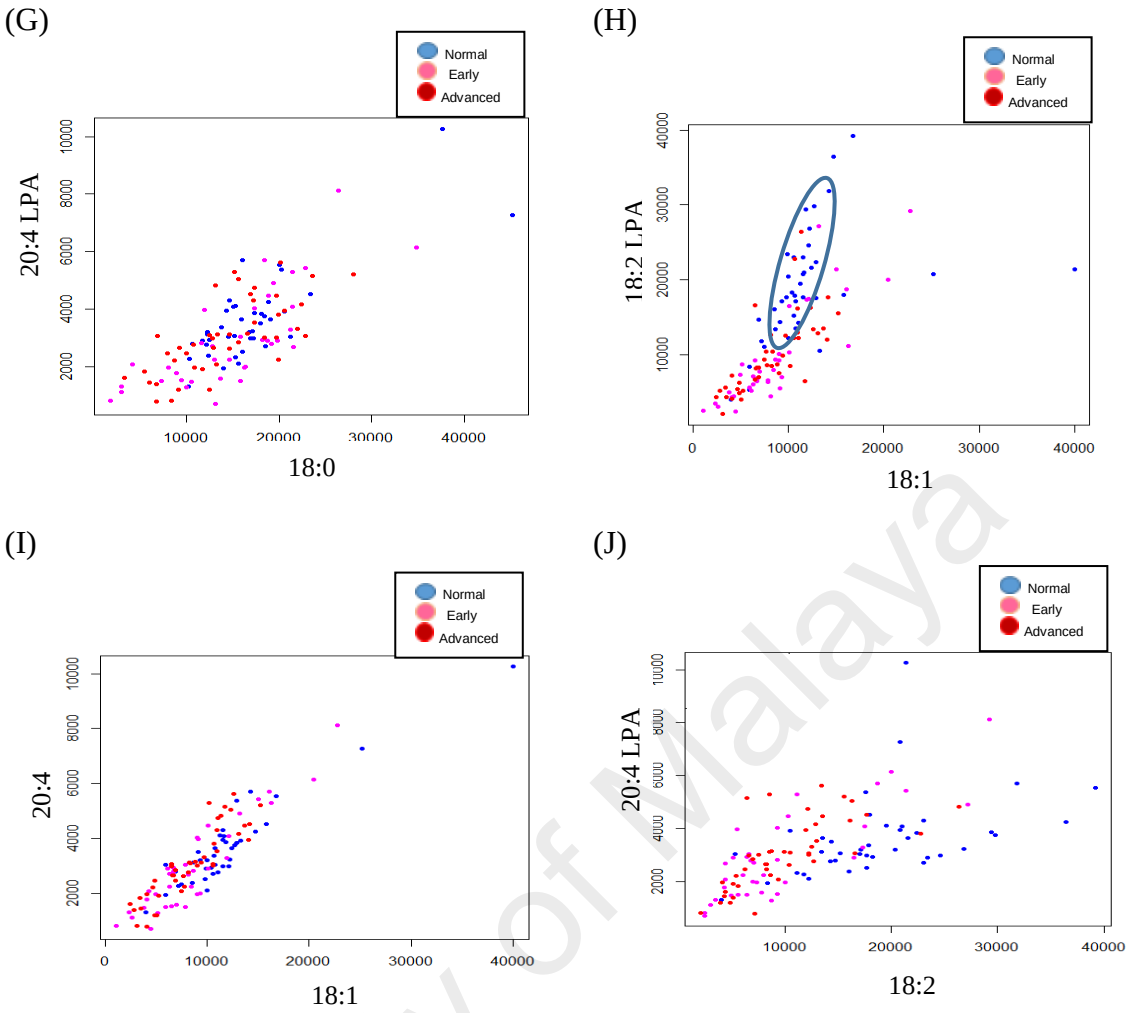



**Figure 6.5G-6.5J: Scatterplots of all possible pairing of five LPA species in three OSCC groups (normal, early and advanced).**
Three pairs that have distinctive differences between the three OSCC groups (normal, early and advanced) are as follows; Figure 6.5C: Pair1- 16:0 LPA versus 18:2 LPA, Figure 6.5F: Pair2 - 18:0 LPA versus 18:2 LPA and Figure 6.5H:Pair3- 18:1 LPA versus 18:2 LPA. In all three figures, normal groups are clustered and labelled with blue ellipse diagram.

### 6.2.6 LPA profiling of OSCC patients and normal controls using heatmaps.

In order to visualise the differences in the levels of all LPA species together, heat maps were generated. Heatmaps are graphical representations of data that utilize color-coded systems. The main purpose of using heatmaps is to better visualize the relative abundance within a dataset and assist in directing towards areas of variation. Basically, it provides one way of visualising patterns in LPA values between different samples and different LPA types. In this analysis, each LPA data has been normalised to the median value of each individual LPA (Figure 6.6).

Heatmaps of early and advanced OSCC groups were different than the normal group. The differences in both groups were characterized mainly by 18:1 LPA, 18:2 LPA and 20:4 LPA.

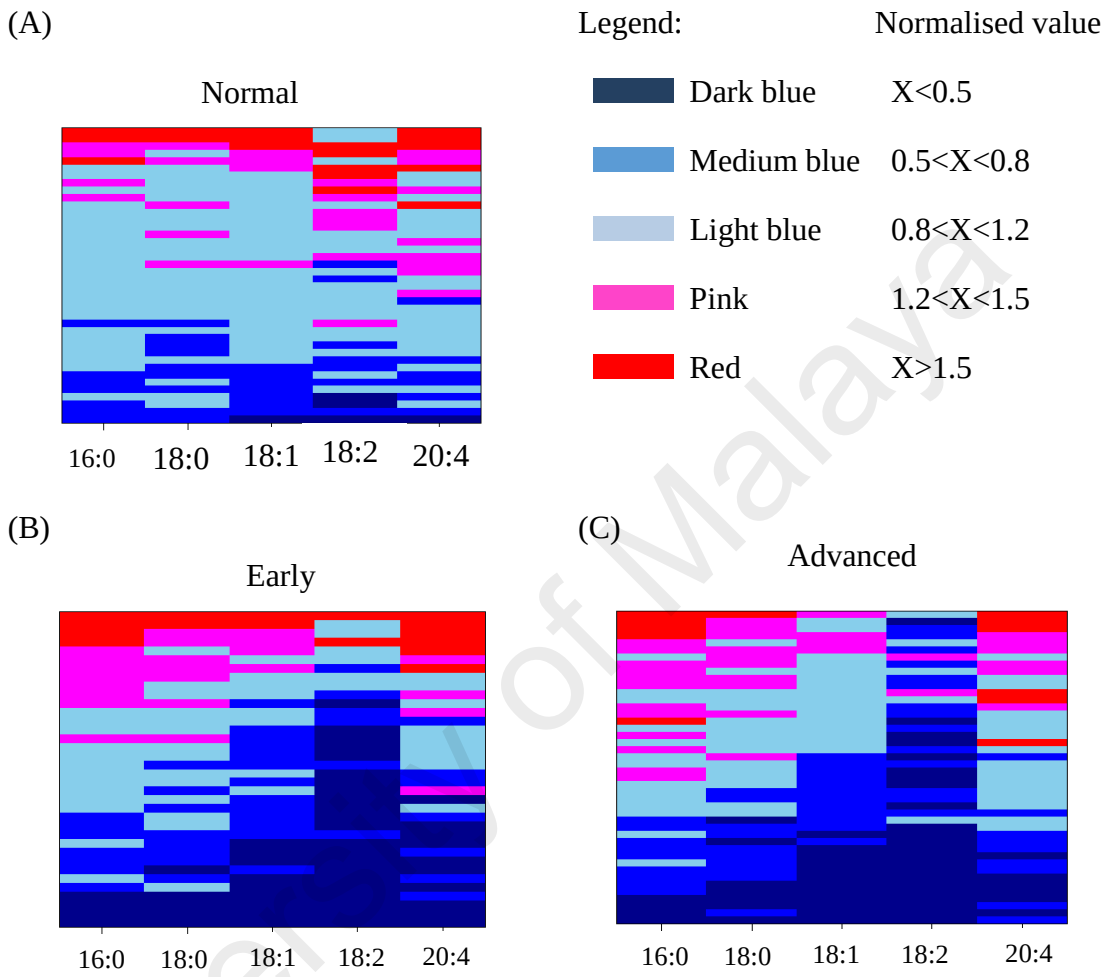



**Figure 6.6: Heatmaps of five LPA levels in normal, early and advanced OSCC groups.**
(A) Normal plasma group (B) Early cancer group (C) Advanced cancer group. Each sample was represented by five LPA values (16:0 LPA, 18:0 LPA, 18:1 LPA, 18:2 LPA and 20:4 LPA) presented in aligned horizontal boxes. In each heatmap, each row represents a different sample, and each column represents a different LPA type. The samples were ordered according to the average of all five LPA values (highest at the top to the lowest at the bottom). For each LPA-type (i.e. each column of the heatmap), the box for each sample is colour-coded according to the ratio of the sample's value divided by the median of all normal values. Light blue box represent the median value for normal sample.

### 6.2.7 LPA profiling of OSCC patients and normal controls using multivariate analysis.

Multivariate analysis is a technique of statistically analysing multiple sets of analytical data. It is a tool to identify patterns and relationships between several variables simultaneously. Principal Component Analysis (PCA) is an example of multivariate analysis. It is a dimension-reduction tool that can be used to reduce a large set of variables to a small set that still contains most of the information in the large set. It transforms a number of correlated variable into a smaller number of uncorrelated variables called principle components.

In order to find any pattern in all LPA levels simultaneously, PCA was performed using all five LPA levels for all 120 OSCC and normal samples (Figure 6.7). The first two principal components (PC1 and PC2) explained the total variation between samples with the values of 78.54% and 13.86% respectively. As shown in figure 6.7A, these two components provide a good separation between the normal samples and both the early and advanced tumour samples. The third and fourth principal components (PC3 and PC4) do not show any clear separation (Figure 6.7B).

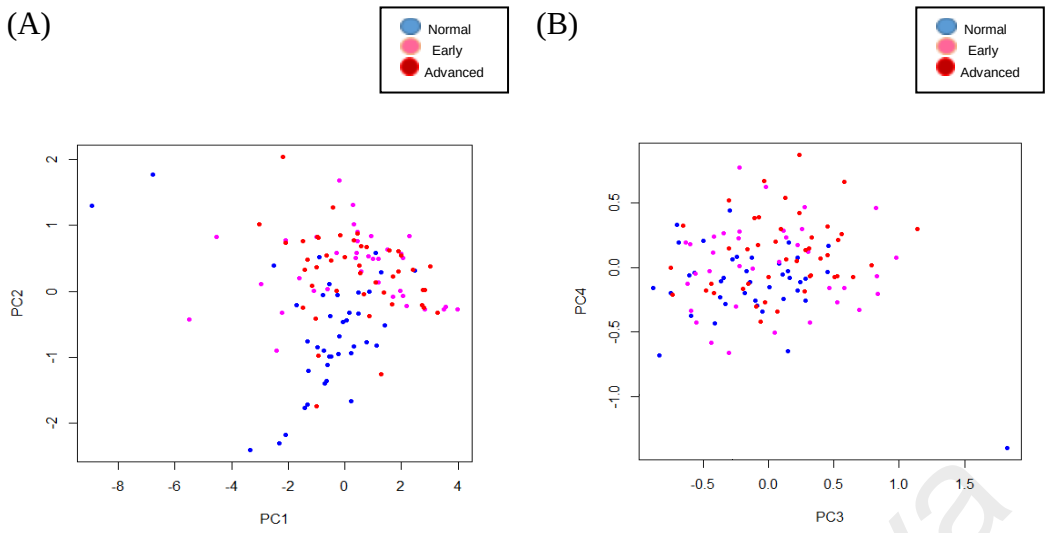



**Figure 6.7: Principal component analysis of abundances of five LPA species in normal and tumour (early and advanced groups) OSCC plasma samples.**
Figure 6.7A: Score plot of PC1 and PC2. Figure 6.7B: Score plot of PC3 and PC4.

## 6.3 Summary

This study was initiated to measure LPA levels in the plasma of OSCC patients and controls. An improved and simplified method to extract and analyse (Section 3.11.2) LPA in plasma using LCMS/MS was developed. Five LPA species (16:0 LPA, 18:0 LPA, 18:1 LPA, 18:2 LPA and 20:4 LPA) were detected and analysed using univariate (boxplots), bivariate (scatterplots) and multivariate analysis (PCA).

Results from boxplots showed that median values of three LPAs, 18:1 LPA, 18:2 LPA and 20:4 LPA in the normal group were higher than early and advanced groups. And the differences between these groups were significant (Kruskal-Wallis test with $p < 0.05$). Kruskal-Wallis post hoc test further identifies significant differences in early-normal and advanced-normal pairs in 18:1 LPA and 18:2 LPA. In 20:4, early-normal pair is also significantly different (post hoc test with Kruskal-Wallis test with $p < 0.05$).

Analysis of the relationship between two LPA species using scatterplots showed normal samples clustered together apart from cancer samples. This means that pairing of

a certain type of LPA allows differentiation between normal and cancer groups. All LPA pairs showed positive correlations using Pearson correlation test.

Heatmaps of OSCC cancer groups showed different profiles when compared to heatmaps of normal samples. In PCA analysis, the first two principal components (PC1 and PC2) explained the total variation between samples provide a good separation between the normal samples and both the early and advanced tumour samples.

In summary, this study has shown that analysing a selected group of LPA species in detail using univariate, bivariate and multivariate enhanced the importance of theses LPA species in profiling and differentiating between normal and OSCC samples. And these results highlight the importance of selected LPA species to become potential biomarkers in plasma samples of OSCC patients.

# CHAPTER 7:

# DISCUSSION

## 7.1 Introduction to Discussion

Ever since the discovery of ATX in melanoma cells as an autocrine motility factor in 1992 (Stracke et al., 1992), the ATX-LPA signalling axis has been associated with the initiation and progression of tumours, survival and metastasis in several cancer types (Benesch et al., 2014) and (Barbayianni et al., 2015). Upregulation of ATX by cancer cells or by adjacent tumour stroma is often accompanied by concomitant overexpression of LPA receptors, particularly LPAR1-3 and a downregulation of ecto-activities of lipid phosphate phosphatase LPP1/3 (Benesch et al., 2014). LPP is an enzyme that plays a big role in clearance of LPA from the circulation. Overall, the net effect of sustained LPA signalling is to increase angiogenesis and cancer growth, migration, and survival (Benesch et al., 2018). Although the ATX and LPA signalling have been studied in a number of major cancers, little is known about its in role in OSCC. Therefore, this study aimed to elucidate the role of ATX and LPA signalling in the pathogenesis of OSCC.

The results from this study showed that the mRNA expression of both *ENPP2* (gene for ATX) and *LPAR3* was upregulated in a proportion of OSCCs, but only the upregulation of *LPAR3* was statistically significant. LPAR3 mediated LPA-induced migration and invasion of OSCC cell lines. LPA was also found to protect OSCC cell lines exposed to low doses of radiation.

In the present study, evidence for possible crosstalk between LPA and EGFR in OSCC cells was inconclusive, as LPA-induced phosphorylation of EGFR was cell line specific. However, LPA was shown to increase COX-2 levels in OSCC cell lines and this was shown to be, at least in part, via LPAR3. Furthermore, LPA-induced cell migration was attenuated by the COX-2 inhibitor, NS398 and COX-2 also plays a role in OSCC cell

survival of LPA-induced resistance to low irradiation doses. These results demonstrate that COX-2 mediates some of the biological effects of LPA in OSCC cells.

Lastly, measurement of LPA levels using LCMSMS in the present study showed that even if individual levels of five major LPA species were lower than normal in the plasma of OSCC patients, the profile of these LPAs as a group analysed with univariate, bivariate and multivariate analysis allowed for the differentiation between normal and cancerous samples.

In this chapter, the discussion subdivided to give consideration to the data presented in each result chapter. The following section discusses the limitations of the study and suggests some possible future directions to take the work forward.

## 7.2 Role of ATX and LPA signalling in OSCC.

The enzyme, ATX, is the protein product of the *ENPP2* gene. ATX converts lysophosphatidylcholine (LPC) into LPA by hydrolysing the choline head group. It was first identified as a tumour motility-stimulating protein, isolated from the conditioned medium of A2058 melanoma cells (Stracke et al., 1992). About a decade later, ATX was linked to LPA production and was shown to be a major producer of circulating LPA levels; LPA levels are reduced by approximately 50% by *ENPP2*-knockout (Umezu-Goto et al., 2002). ATX is highly expressed in several different cancers, for example, osteosarcoma, melanoma and neuroblastoma. However, acute lymphoblastic and myeloic leukaemias and colon carcinomas appear to be characterised by relatively low ATX expression (Willier et al., 2013). Currently, its expression in OSCCs is unknown. However, ATX mRNA has been reported to be upregulated in 46% (7/15) of micro-dissected NPCs, a sub-group of head and neck cancer (Yap et al., 2015).

### 7.2.1 Expression of ATX in OSCC.

Several mechanisms have been proposed to explain the overexpression of ATX in cancer. Apart from copy number amplification, enhanced ATX mRNA stability by RNA binding protein such as human antigen R (huR) in melanoma cells has been shown to increased ATX production (Sun et al., 2016). In thyroid cancer, sustained autocrine-induced inflammation upregulated ATX by a mechanism that could the overexpression of huR (Benesch et al., 2015). Breast cancer also express high levels of ATX (Yang et al., 2002). However, some breast cancer cells secrete little ATX compared to the basal rate from breast adipose tissue (Benesch et al., 2015) and these authors have uncovered a paracrine-model of ATX production in breast tumours. Secretion of inflammatory mediators from the breast tumour induces ATX expression in adjacent mammary tissue which increases LPA within tumour microenvironment. Despite the fact that even though some tumours express high levels of ATX as in the case of breast cancer, LPA levels may still be locally increased even when tumour cells do not obviously over-express ATX. In the present study, there was no significant difference in the expression of ATX between OSCC and its normal counterparts, although up-regulation of ATX mRNA was clear in a sub-set of tumours, results which demonstrate the heterogeneous nature of OSCC. It would be interesting to see whether the secretome of OSCC cells could induce ATX expression in adjacent cells or tissues.

### 7.2.2 Expression of LPA receptors (LPARs) with a focus on LPAR3.

Different cancers display distinct expression signatures of LPARs that distinguish them from one another. For instance, LPA receptors were expressed in a variable degree in colon, breast, melanoma and lung cancer cell lines tested (Muller et al., 2010). Another study also showed that the expression LPA receptors was heterogeneous, such that colon carcinomas feature upregulation of *LPAR3* and *LPAR5* genes, whereas all other LPARs

were downregulated. Ovarian carcinoma also displayed upregulation of all LPARs except for *LPAR5*, whereas stomach carcinomas show the opposite LPAR pattern concerning *LPAR3* and *LPAR5* (Willier et al., 2013).

The results from the present study also showed that the expression of LPARs was variable and unique to each OSCC cell line. It was found that all LPARs except *LPAR4* were expressed in all OSCC cell lines, with the exception of H376. Variable gene expression is to be expected when analysing various cancerous cell lines. In OSCC tissues, *LPAR3* mRNA expression was significantly upregulated compared to normal controls. It should be noted that a subset of tumour samples showed considerably higher expression compared to other tumour samples suggesting that mRNA expression also varies greatly in the tumour group samples.

### 7.2.3 Associations between ATX and LPAR3 with clinic-pathological parameters.

In human breast cancer, increased ATX expression in breast tumour epithelium is associated with increased tumour size and advanced disease stage (Popnikolov et al., 2012). In the present study, however, even though the mRNA levels of *ENPP2* in OSCC tissues were not significantly higher than the normal samples, there were still significant associations of *ENPP2* gene with betel nut chewing and cancer stage.

The mRNA expression all LPARs have been analysed in this study. Although only *LPAR3* gene expression was significantly upregulated in OSCC, there was a significant correlation between *ENPP2* and *LPAR3* genes. The Spearman rank correlation indicates a direct, positive relationship. This suggested that as one of the gene expression increases, the expression of the other gene tend to go in the same direction. Further work is required to elucidate the precise contributions of both ATX and LPAR3 in oral carcinogenesis.

Interestingly, both *ENPP2* and *LPAR3* mRNA levels showed a significant association with betel nut chewing in OSCC patients suggesting novel roles for *ENPP2* and *LPAR3* in betel-nut-induced carcinogenesis. There is sufficient evidence to link betel nut chewing

with or without added tobacco with oral cavity, pharynx and oesophagus cancers (Secretan 2009). However, the underlying mechanisms linking the betel nut chewing to oral carcinogenesis is still not well understood. The link between both ATX and LPAR3 with betel nut chewing should be further investigated as it has been proposed that OSCC induced by tobacco smoking and betel nut chewing has a distinct molecular basis (Saeed et al., 2015).

### 7.2.4 Role of LPA and LPAR3 in motility of OSCC.

Metastasis is defined as a process where genetic instability in the primary tumour fuels cell heterogeneity, allowing for a few metastatic clones to eventually emerge to be positively selected to disseminate cancer at a distance (Klein, 2009). Metastasis consists of sequential and selective steps including proliferation, stimulation of angiogenesis, detachment, motility, invasion into bloodstream and crosstalk with components of the new microenvironment (Fidler, 1990; Nguyen et al., 2007). The current study chose to focus on cell motility because it is closely involved in invasion and metastasis during the progression of cancer (Wells et al., 2013). Various LPA receptors have been implicated in LPA-induced cell migration depending in the tumour type (Sengupta et al., 2004). It is interesting to note that each LPA receptor exhibit diverse effects in cell motile activities of cancer cells. Human colon carcinoma with high expression levels of LPAR1, low expression of LPAR2 and none for LPAR3 showed enhanced cell migration, proliferation and adhesion (Shida et al., 2003). LPA also induced marked migration of gastric cancer cells that expressed significant levels of LPAR1 but not cells with LPAR2 (Shida et al., 2004). On the other hand, neuroblastoma cells expressing only LPAR1 showed markedly decreased cell motility and invasion, which were slightly enhanced by LPA treatment (Hayashi et al., 2012). LPAR1 knockdown cells of pancreatic cancer cells showed enhanced cell motility and invasion whereas, LPAR3 knockdown cells showed

significantly lower cell motility and invasion (Kato et al., 2012). Activation of LPAR2 and LPAR3 strongly stimulates cell motility in ovarian cancer cells (Yu et al., 2008).

In the present study, the effect of exogenous LPA on cell migration and invasion were investigated *in vitro.* LPA was shown to induce migration of OSCC cells in transwell migration assays in a dose-dependent manner. H357 required a minimum concentration of 10μM LPA for a significant cell migration result whereas H376 required a range of between 2.5-10 μM LPA to achieve similar results. These results showing differential concentrations of LPA in different OSCC cell lines is similar to a study by Brusevold, et al. (Brusevold et al., 2014). In their study, they have also showed a dose-dependent wound closure with LPA stimulation in three OSCC cell lines using 10 μM of LPA resulting in the maximum effect in the wound scratch assay.

Increased expression of LPAR3 and ATX in human breast cancer is associated with tumour aggressiveness (Popnikolov et al., 2012). LPAR3 enhanced invasive activities of neuroblastoma and hepatoma cells (Hayashi et al., 2012) and (Okabe et al., 2013). Following the demonstration that LPAR3 is overexpressed in OSCC, the role of this receptor in mediating LPA-induced cell migration and invasion was investigated *in vitro*. LPA-induced cell migration was attenuated by 10μM of Ki16425 (LPAR1/3 antagonist) in both OSCC cell lines (H357 and H376). These results suggest that activation of LPAR1 and/or LPAR3 play an important role in OSCC cell migration. Knockdown of LPAR3 in H357 and BICR31 also resulted in a significant reduction of LPA-induced cell migration, thereby confirming a role for LPAR3 in this process. Preliminary data from our laboratory indicates that LPA-induced cell migration on OSCC cell lines is through activation of Rac, ROCK and MAPK pathways (Johnson and Paterson, unpublished observations). Taken together, these results suggest that LPA-induced cell migration in OSCC occurs via the activation of Rac, ROCK and MAPK pathways via LPAR3.

Inhibition of LPAR1/3 by Ki16425 also inhibited invasion of H357 cells in transwell invasion assay. In this assay, cells must be able to invade through a thin layer of Matrigel Basement membrane matrix. This matrix is composed of laminin, Collagen IV, nidogen/enactin and proteoglycans, which mimics the basement membrane. The ability of Ki16425 to inhibit the invasion induced by LPA supports a role for LPAR1 and/or LPAR3 activation in the invasive ability of OSCC cells.

### 7.2.5 Role of LPA and LPAR3 in protection against irradiation.

Another important factor that has been linked to the LPA/LPAR axis is the resistance of tumour cells to radiation. Currently radiation therapy (RT) is a major and standard local treatment for OSCC patients. However, sometimes the development of radio-resistance results in treatment failure. Effective radiotherapy principally depends on successful induction of apoptosis (Verheij et al., 2000). There are accumulating evidences for the role of the LPA/LPAR axis in radio-resistance where it might protect and rescue various cancer cells from radiotherapy-induced apoptosis.

A previous study has reported that LPA rescued non-transformed intestinal crypt-derived epithelial cells from apoptosis when administered one hour before or two hours after high dose irradiation (>10 gray (Gy) (Deng et al., 2002). Later, they reported that anti-apoptotic effect of LPA was due to specific activation of LPAR2 (Deng et al., 2007). Another study utilising a non-lipid agonist specific to LPAR2, GRI977143, rescued apoptotically condemned cells treated with high dose γ-irradiation (Kiss et al., 2013). These studies demonstrated the protective effects LPA and LPAR specifically LPAR2 against high doses of irradiation.

In the present study, the protective effect of LPA on OSCC cell lines exposed to irradiation was tested using clonogenic survival assays lasting 12 days. Preliminary experiments were conducted and the results showed that less than 50% of the cells

survived radiation doses within the range of 2-8 Gy. Therefore, only doses of one and two Gy were selected for the radiation treatment in order to get viable cell colonies. In the clinical settings, the international standard for definitive treatment remains as 70Gy in daily fractions of 2Gy over seven weeks for head and neck cancer (Radiotherapy dose fractionation, 2nd edition 2017). Therefore, in general, the experimental treatment doses used in the present study are clinically relevant.

Pre-treatment with LPA for one hour or overnight have increased the percentage of cell survival significantly compared to no-treatment group after exposure to low doses of irradiation (one or two Gy) on both OSCC cell line (H357 and BICR31). When the cells were pre-treated with the LPAR1/3 inhibitor Ki16425, the protective effect of LPA was decreased. However, this effect was only apparent after the exposure to one Gy irradiation and only in one OSCC cell line (H357). No protective effect was detected when the experiment was repeated with another OSCC cell line BICR31.

Even though both cell lines originate from the tongue, H357 was shown to have higher gene expression of *LPAR3* compared to BICR31 (Figure 4.3). This could be the reason why Ki16425 was only able to counter the protective effect of LPA against low doses of irradiation in H357 cells.

Preclinical and clinical evidences have highlighted three main pathways that play crucial roles in radioresistance development in HNSCC (Perri et al., 2015). These pathways are the EGFR, the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamysin (mTOR) and the p53signalling cascades. In relation to the results of my study, further research should focus on the activation of PI3K/AKT/mTOR pathway via LPAR1/3 coupled with GCPR, Gαi/0 to confirm the signalling pathway (Figure 7.1)(Yung et al., 2014).

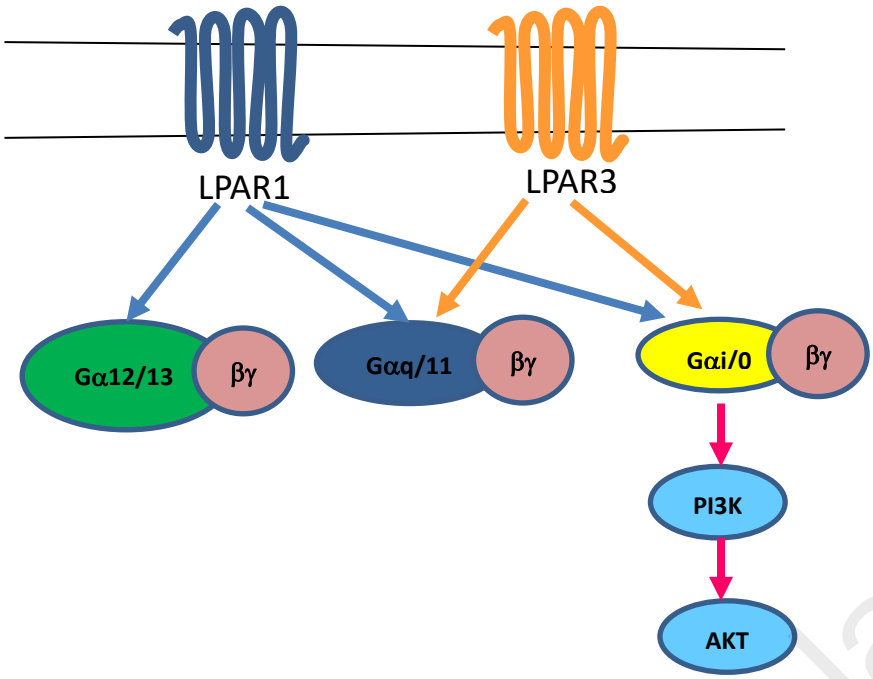



**Figure 7.1: Activation of PI3K/AKT/mTOR signalling pathway via LPAR1/3 coupled with GCPR, Gαi/0.** Adapted from (Yung et al., 2014)

### 7.3 Crosstalk between LPA, EGFR and COX-2 and its Functional Significance in OSCC.

Overexpression of EGFR has been detected in OSCC. Positive EGFR and pEGFR staining was pres ent in 92.3% (48/52) and 98.0% (51/52) of all OSCC tissue samples (Hiraishi et al., 2006) (Sarkis et al., 2010). EGFR overexpression has been correlated to poor prognosis in OSCC patients (Oliveira et al., 2011) and (Aquino et al., 2012). Upregulation of EGFR has been implicated in the progression of OSCC. Earlier study has showed that EGFR in normal epithelia adjacent to the tumours was 2-fold higher than in normal controls. Further upregulation of EGFR expression occurred in the change from dysplasia to squamous cell carcinoma (Shin et al., 1994). Another study showed that EGFR expression was a good predictor for the progression of hyperplasia to dysplasia, and in the progression into invasive carcinoma (Shah et al., 2007).

Overexpression of COX-2 has also been detected in OSCC. A number of studies have showed that COX-2 expression levels were significantly higher in OSCC compared to

normal control human tissue samples ((Moazeni-Roodi et al., 2017), (Lee et al., 2015) (Byatnal et al., 2015; Goulart Filho et al., 2009) and (Tang et al., 2003). Upregulation of COX-2 has been implicated in the progression of OSCC. COX-2 mRNA and protein were absent in normal oral mucosa but were expressed in increasing intensity as oral epithelial dysplasia progressed to OSCC (Nagatsuka et al., 2012). A positive correlation was found between COX-2 expression and severity of dysplasia (Seyedmajidi et al., 2014). These data suggests that both EGFR and COX-2 are important markers of early malignant transformation and progression of oral cancer.

A number of studies have showed that COX-2 is a downstream mediator of LPA signalling pathways as discussed in section 1.12. Activated EGFR leads to phosphorylation of tyrosine residues by the receptor's tyrosine kinase domain, initiating MAPK signalling pathway which further activates transcription factors such as c-myc, c-fos and c-jun (Gupta et al., 2000) and expressed COX-2 (Figure 7.2). C/EBP is another transcription factor that mediates COX-2 expression in ovarian cancer cells (Oyesanya et al., 2008).

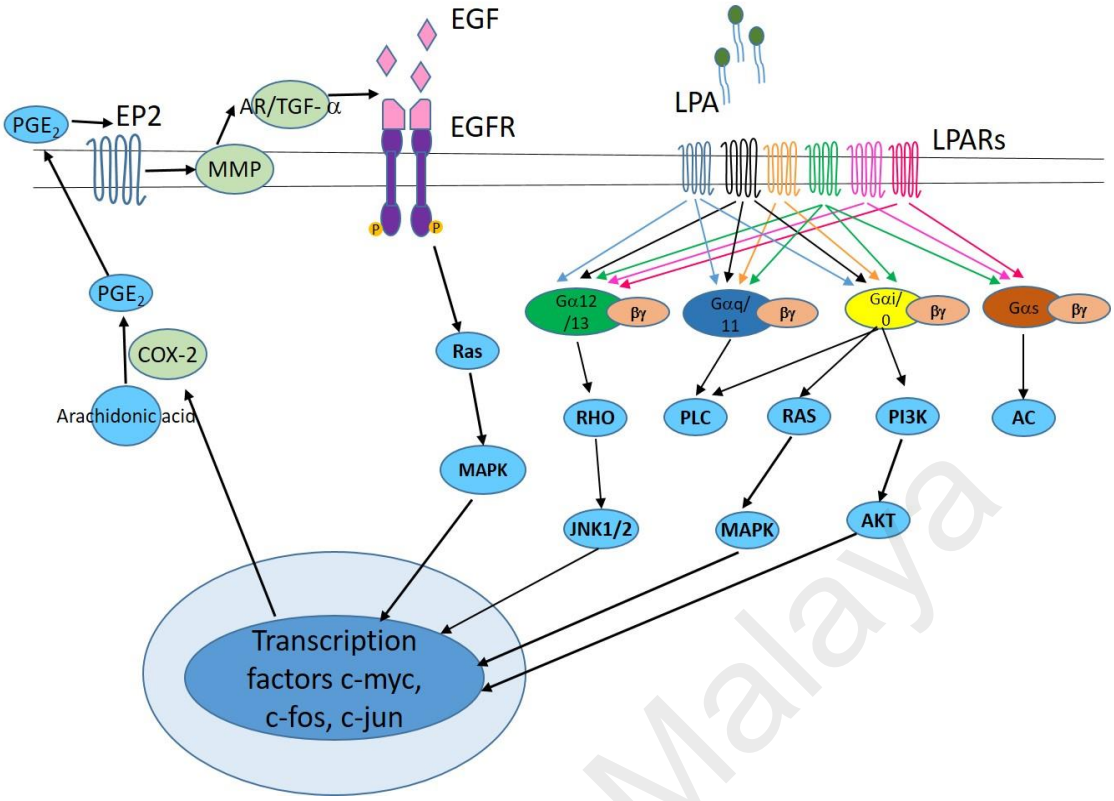



**Figure 7.2: Combination of EGFR, LPA-GCPR and COX-2 pathways.**
Binding of LPA to the LPA receptors, activates the G proteins which in turn will stimulate the release of α subunits from the heterodimeric composition. Independent α and β+γ pair can each proceed to regulate enzymes that evoke a variety of downstream responses including COX-2 expression. In the middle of the diagram, activation of EGFR activates MAPK which results in activator protein-1 (AP-1)-mediated induction of COX-2 transcription and enhanced synthesis of PGE-2. MAPK pathway also activates a group of nuclear transcription factors (c-myc, c-fos and c-jun) which also induce transcription of COX-2. PGE-2 then can activate EGFR signalling by binding to PGE-2 receptor (EP2). EP2 can promote the transactivation of EGFR (expressed in colon cancer cells) through Src which activates proteolytic release of EGFR ligands amphiegulin (AR) and transforming-growth factor-α (TGF-α), thereby stimulating the EGFR-signalling network and creating a loop in cancer development. Adapted from (Gupta et al., 2000) and (Dorsam et al., 2007).

In this study, the crosstalk between LPA, EGFR and COX-2 signalling pathways was examined in OSCC cell lines. When the OSCC cell lines were treated with LPA, there was no clear phosphorylation of EGFR. This may be due to the high basal phosphorylation that had already existed before the LPA treatment. This result was similar to the previous study by (Hiraishi et al., 2006) and (Sarkis et al., 2010) where most of their OSCC tissue samples showed high expression levels of both EGFR and

pEGFR. If there are any subtle differences between total and phosphorylated EGFR, other techniques such as the use of mass-spectrometry should be used in the future in order to detect any differences to confirm any interaction. It will still be beneficial and a challenge to understand the molecular mechanism underlying the transactivation of LPA and EGFR in cancer and develop therapies to target GPCR-mediated transactivation.

### 7.3.1 LPA induces COX-2 expression in OSCC cell lines.

In the present study, COX-2 protein levels were clearly increased in OSCC cell lines when treated with LPA. Previously, LPA has been shown to induce COX-2 expression in human colon cancer cells (Shida et al., 2005a). In ovarian cancer, LPA affected the transcription activation and post-transcriptional enhancement of COX-2 mRNA stability (Oyesanya et al., 2008). When the OSCC cell lines were pre-treated with LPAR1/3 inhibitor, Ki16425 prior to stimulation with LPA, COX-2 expression was attenuated. This result suggests the involvement of LPAR1/3 in mediating this effect. To the best of my knowledge this data represent the first instance where LPAR1/3 is involved in inducing COX-2 expression in OSCC cell lines. This result is supported by the decreased COX-2 levels in LPAR3-shRNA transfected OSCC cell lines after two hours of LPA treatments. These data demonstrate the involvement of LPAR3 in regulating the levels of COX-2 in OSCC cell lines.

### 7.3.2 COX-2 inhibitor inhibits LPA-induced OSCC cell migration

A large number of studies have focused on the role of LPA signalling in the regulation of GPCRs that leads to cell migration, cancer cell invasion and metastasis. However, only a few studies have investigated the effect of LPA on acquisition of the migration phenotype mediated via a COX-2 dependent mechanism. COX-2 played a role in LPA-induced migration where treatment with COX-2 specific inhibitor NS-398 reduced

ovarian carcinoma cell migration ((Symowicz et al., 2005). Another study reported that LPAR2 and Gi/Src pathway mediated cell motility through COX-2 expression in CAOV-3 ovarian cancer cells (Jeong et al., 2008). In the present study, LPA-induced migration of OSCC cell lines was significantly attenuated following COX-2 inhibition with NS-398. Since both LPAR2 and LPAR3 are coupled to one of the G-coupled protein receptor, Gi, further studies should be carried out to investigate whether COX-2 related migration in OSCC cell lines has similar downstream activation via Gi/Src pathway as reported in ovarian cancer cells (Jeong et al., 2008).

### 7.3.3 COX-2 is involved in LPA-induced radioresistance in OSCC cell line.

Overexpression of COX-2 has been correlated with poor prognosis in squamous cell carcinoma of uterine cervix treated with radiation therapy (Gaffney et al., 2001). In OSCC, overexpression of COX-2 was associated with radioresistance (Terakado et al., 2004). Their study reported that radiation sensitivity of eight OSCC cell lines differed greatly in response to irradiation. Our results also showed similar trend where only one cell line showed significant differences of the survival fraction compared to control in response to irradiation. When OSCC cell lines were pre-treated with LPA and COX-2 inhibitor NS398, there were significant differences of the survival fraction compared to control group (without NS398 treatment). This result demonstrated that the COX-2 is involved in the survival of OSCC cells induced by LPA. In spite of that, the survival effect is cell specific. This study further supports the important role of COX-2 in radioresistance where its gene *PTGS2* was among the 25 genes reported to be associated with proliferation and anti-apoptosis in cancer (Ishigami et al., 2007).

## 7.4 The Potential of LPA Plasma levels as a Biomarker in OSCC.

### 7.4.1 Challenges in LPA extraction and LCMSMS analysis.

Various methods have been employed previously to measure LPA levels in biological fluids. Some of the earliest methods used were by direct and indirect detection in the plasma samples. Indirect detection was been carried out using bioassays with voltage-clamped xenopus oocytes. Although this method was highly sensitive, it lacked the ability to distinguish compound classes or molecule structures. Another indirect method was to detect LPA-derived fatty acid methyl esters using thin layer chromatography (TLC) and gas chromatography mass spectrometry (GCMS). In these methods, LPA was chemically modified by hydrosylation followed by methylation. Total LPA detected in ovarian cancer samples using TLC-GCMS showed increased levels (8.6μM) as compared to healthy controls (0.6 μM) (Xu et al., 1998). They also reported that nine out of ten samples from stage I and all cancer samples from stage II, III and IV have elevated levels of plasma LPA. Other gynaecological cancer samples (33/36) also had higher LPA levels as compared to normal samples. In their study, CA 125 was also detected in the same samples. Currently, CA 125 is the only plasma tumour marker routinely used to monitor disease progression, patient's prognosis and response to chemotherapy (Bast et al., 2002; O'Brien et al., 1998). CA 125 is a membrane glycoprotein produced by tissues derived from the coelomic epithelium that is expressed by most epithelium ovarian cancers. Only 28/47 ovarian cancer plasma samples have elevated CA125 levels including 2/9 stage I patients. In comparison with the CA 125 levels, plasma LPA may represent a potential biomarker for ovarian cancer (Xu et al., 1998).

Problems that arose from these techniques are the absence of appropriate standards for product recovery throughout the procedures. Various LPA salts that were produced in these procedures differ in mobilities via TLC. Different types of TLC plates (acidic, basic

and neutral) also caused different rate of mobilities. These problems would lead to the underestimation of LPA levels (Baker et al., 2000). A radioenzymatic assay together with use of $^{14}$C-labelled PA separated by TLC and detected by autoradiography has been reported (Saulnier-Blache et al., 2000). Although this method was more simple and sensitive, it requires the use of radioactivity and it was still unable to differentiate between all available LPA species.

To overcome these problems, a more direct method was chosen. Development of LPA analysis using LCMS grew over the years. Baker et al (2000) developed a stable-isotope-dilution, negative-ion electrospray-ionization liquid chromatography (LC) mass spectrometry to directly quantify LPA in biological samples. Normal phased column was used in this analysis. They selected and measured 5 individual LPA acyl species (16:0 LPA, 18:0 LPA, 18:1 LPA, 18:2 LPA, and 20:4 LPA) which comprise of more than 90% of total plasma LPA. A high throughput LC ESI/MS/MS employing reversed phase C18 column has been utilised to analyse LPA levels resulting in the following orders 18:2 LPA>16:0 LPA, 20:4 LPA>18:1 LPA, 22:6 LPA and 18:0 LPA (Shan et al., 2008). LPA and sphingosine-1-phosphate (S1P- another lipid signalling molecule) were quantified in a faster analysis time of 2.5mins using another type of LC called hydrophilic-interaction LCMS/MS (Scherer et al., 2009). Utilising LC prior to MS was highly recommended together with improved sample extraction procedure (Bollinger et al., 2010).

In the present study, five major LPA found in the plasma were selected, as suggested by Baker et al., (2000). Initially, the LPAs were separated with a reversed phase C18 column. However, I encountered carry over effects in the runs. The column was changed to a C6 column, which resulted in shorter, cleaner and more successful runs. Due to limited amount of samples available, a simple extraction method was adopted (Zhao et al., 2010) using a single MeOH solvent as it is also compatible with ESI/MS. This method

allows the extracted lipid to be used directly where the usual solvent evaporation steps could be eliminated.

The range of total LPA concentrations in normal samples detected by various published mass spectrometry protocols is between 0.61 to 5.1 µM (Table 7.1). This study showed that LPA concentrations were approximately 28 times higher in normal plasma compared to other studies listed in Table 5.3. This result may be due to the different lipid extraction protocol, type of column and running condition in the chromatography and the mass spectrometry analysis. In my study, LPA was extracted using a modified and simple method for LPA extraction as described by Zhao and colleagues (Zhao et al., 2010). This method extracts LPA by precipitating blood proteins in 150µL of methanol. There is no solvent evaporation step. Therefore, extracted lipids can be directly used for the analysis. Due to the limited volume of plasma samples available, this modified method uses only 10uL of plasma which was added to 300uL of methanol. This method only uses one solvent and one step of centrifugation, which saved the volume of solvents used in the running of the chromatography and the analysis time.

**Table 7.1: Different extraction methods with LPA concentrations in healthy control samples determined by mass spectrometric analysis.**

| Plasma | Lipid extraction | Method of analysis | Total LPA concentrations | Reference |
|---|---|---|---|---|
| Pooled EDTA plasma (male)<br>Pooled EDTA plasma (female) | Butanol and citrate-phosphate buffer (pH4) | LCMS | 0.61± 0.14 µM<br>0.74 ± 0.17 µM | (Baker et al., 2001) |
| Plasma | Acidification<br>Bligh & Dye's method $CHCl_3/CH_3OH$ extraction | Direct injection to ESI MS/MS | 4.3-5.1 µM | (Yoon et al., 2003) |
| EDTA plasma | Acidification<br>Bligh & Dye's method $CHCl_3/CH_3OH$ extraction | LC-ESI MS/MS | 0.95-2.03 µM | (Shan et al., 2008) |
| EDTA plasma | Modified Bligh & Dye's method with butanol extraction | LCMS/MS | 0.70± 0.20 µM | (Scherer et al., 2009) |

Although the results from this study showed that individual or total LPA levels were higher than other studies, the profile or order of abundance of LPA species was similar for the normal plasma samples. The order of abundance of the LPA species found in normal plasma samples was as follows: LPA: 18:2>16:0>18:0>18:1>20:4 which is similar to another study by Shan et. al. where both 18:2 LPA and 16:0 LPA were also found to be among the highest concentration following the same order (Shan et al., 2008). Baker *et. al,* 2002 also observed a similar pattern where 18:2 LPA was the highest concentrated LPA species, followed by 20:4 LPA, 16:0 LPA,18:1 LPA and 18:0 LPA in normal samples (Baker et al., 2002). The profiles for both cancer groups were different than the profile of normal plasma samples. The profiles of LPA species detected in both early and advance cancer groups were the same which is LPA 16:0>18:0>18:2>18:1>20:4.

Another important advantage of my analysis is that for every LPA quantitation, only strong correlation coefficient values (r) were accepted while generating the standard curve. A perfect correlation is when the r value is 1 and standard curves were only accepted if r >0.995 in my study. Standard curve points of pure LPA were prepared in blank plasma (without any LPA) and were then extracted using the same extraction method. A linear regression standard curve would be impossible to be generated if there were problems with the extraction, separation and analysis methods.

### 7.4.2 Profiles of LPA species in univariate, bivariate and multivariate analysis.

When comparing the LPA levels (individually or in total) in cancer versus normal samples, promising results have been shown in ovarian cancer studies (Cao et al., 2015; Zhang et al., 2015). However, contradictory results have been published by Baker et al 2002 who reported that there were no significant differences in the concentrations of either individual LPA values or total LPA in the plasma of ovarian cancer patients compared to normal controls (Baker et al., 2002). In the present study, no increase in LPA levels were observed in plasma from OSCC patients, results that concur with the study by Baker et al. 2002. However, even though the LPA levels in OSCC plasma samples were lower than the normal samples, the ranking and order of the concentration of the five LPA species were different from the normal group.

This observation could be due to the results from the previous section where the gene expression of *ENPP2* for the enzyme ATX that produces LPA was found to be low in OSCC cell lines. Another possibility could be increased activity of lipid phosphate phosphatase (LPP) which is the enzyme that plays a major role in clearance of LPA from the circulation. Concentrations of the major LPA species in mouse plasma decreased uniformly following administration of potent selective inhibitor of LPA-generating lysophospholipase D ATX identifying an active mechanism for removal of LPA in circulation (Salous et al., 2013). There are three LPP isozymes (LPP1-3) which originate from a widely expressed LPP family of genes. The disappearance of intravenously administered LPA in homozygous mice with a disrupted LPP1 gene *(PPAP2A)* demonstrated that LPA was rapidly metabolized in the bloodstream (Tomsig et al., 2009). Overexpression of LPP1 in breast and thyroid cancer cell lines decreases tumour growth and metastasis by up to 80% as compared to the expression of catalytically inactive LPP1 in both syngeneic and xenograft mouse models (Tang et al., 2014). Another evidence

came from a study where low expression levels of LPP1 gene has been identified as one of the twelve predictive markers of poor breast cancer survival. Similarly, by increasing the low levels of LPP3 was shown to decrease the growth, survival and tumorigenesis of ovarian cancer cells (Tanyi et al., 2003). LPP2 however exerts different effects than LPP1 and LPP3. LPP2 expression was found to be increased in breast, lung and ovarian cancers, as opposed to LPP 1 and LPP3 expression (Tang et al., 2015). Further work should be conducted in the future to investigate the LPP levels in OSCC.

To the best of my knowledge, this is the first study to analyse LPA levels in OSCC plasma samples. In this study, even though LPA levels in OSCC plasma samples were detected lower than normal samples, there were differences between the sample groups when analysed using univariate, bivariate and multivariate analysis.

In the first box-plot comparison between all three plasma sample groups, normal, early and advanced groups,18:1 LPA, 18:2 LPA and 20:4 LPA levels were found to be significantly different the (Kruskal-Wallis test ($p$<0.05). Further testing using post hoc test identified that significant differences were between normal and early groups for all three individual LPA species.

To study the relationship between two LPA species, scatterplots were constructed by pairing all LPA species together. Results from scatter plots showed that when analysed with Pearson correlations test, all pairs demonstrated positive and significant differences between two LPA species tested. Clusters of normal samples were separated from both the early and advanced cancer groups in three pairs. The pairs are 16:0 with 18:0 LPA, 18:0 with 18:2 LPA and 18:1 with 18:2 LPA. These results suggest that the pairs may be exhibiting a more, or less aggressive phenotype that could lead to metastasis.

These results mean that as the value of one LPA increases, the other LPA species will also increase. This could relate to the presence of ATX and the availability of the precursors for LPA synthesis. Further studies should continue on the regulation of LPA synthesis especially in systemic circulation or at the local setting of the tumour microenvironment.

When the profiles of LPA levels were generated using heatmaps, clear differences between the three groups were observed. Multivariate analysis using principle component analysis (PCA) results also provides good separation between the normal and both early and advanced cancer groups. Profiling groups of samples in lipidomics has two categories. The first category is exploratory and PCA falls under this category. Explorative study is used to see whether the specific data contains enough information to make a distinction between diseased and control groups. The second category is predictive category where a predictive model is generated and it is used to predict whether an unseen individual or sample belongs to the case or control group (Westerhuis et al., 2008). An example of the predictive model is called Partial Least Squares Discriminant Analysis (PLS-DA). One of the potential uses of this model is to predict OSCC samples that may have the profile of becoming more aggressive than the non-aggressive type especially at the early stages of cancer.

In summary, profiling of the five major LPA species was able to separate or differentiate OSCC than the normal control samples in all three types of statistical analysis. Profiling individual LPAs might not show any differences but more information can be gathered when analysing the LPAs as a group. These results highlight the importance of selected LPA species to become potential biomarkers in OSCC.

Currently, several studies have been conducted on the biological roles of individual LPA species. Unsaturated LPA species (16:1 LPA, 18:1 LPA and 18:2 LPA) induced

marked morphological changes of vascular smooth muscle cells and suppressed the gene expression of differentiation markers compared to the saturated LPA species (12:0 LPA, 14:0 LPA, 16:0 LPA and 18:0 LPA) (Hayashi et al., 2001). Unsaturated LPAs (18:1 LPA and 20:4 LPA) were more potent than saturated LPA (16:0 LPA) in stimulating cell growth in one ovarian cancer cell line (SKOV-3) through LPAR3, whereas, all three LPAs were equally potent in Caov-3 cell growth (Hurst et al., 2009)._Unsaturated LPA species were reported as more efficient chemoattractants than unsaturated LPAs in immature mouse dendritic cells mediated by LPAR3 (Chan et al., 2007). In summary, these studies showed that unsaturated LPAs play a role in changes in cell shape, cell growth and migration. Since the altered LPA profiles of OSCC in the box-plot analysis in the present study were all unsaturated LPAs, further studies to investigate whether these specific LPA species modulates the migration, invasion and radioresistance are warranted.

### 7.5 Study Limitations

The experiments in this study have been carefully planned to answer generally the hypothesis and specifically all the objectives of the research questions. However, there were some limitations that have been encountered during the course of this work.

Firstly, in quantitative research, the number of samples plays a big role in the analysis. Small sample sizes will make it difficult to find significant relationships because statistical test normally would require a large sample size in order to ensure a representative distribution of the population. In this case, the bigger the number of samples, the better the results will be. Calculation for the optimum sample size have been applied for example, the quantitation of LPA levels in the plasma of OSCC patients. In the future however, increasing the number of plasma samples would be better if the experiments were to be repeated with other cancer types.

Secondly, in the present study, only mRNA upregulation of *LPAR3* was reported in the OSCC tissue samples. I attempted to detect LPAR3 protein expression in tissue samples using immunohistochemistry however there were problems with non-specific binding from the antibodies tested. Commercialised antibodies should be chosen with caution in order to get quality results.

Thirdly, it is important to note that the treatment of inhibitors in the *in vitro* assays were carefully selected and optimized to specific OSCC cell lines. Special consideration should be taken when testing them in *in vivo* situations.

### 7.6 Future Directions

The results from the present study have identified some potential areas that can be further explored in the future. This is to get more information and knowledge that would really enhance our understanding of the role of the LPA signalling pathway in carcinogenesis.

Firstly, the result from chapter 4 showed that gene expression of *ENPP2* of the OSCC tissue samples was not significantly different than normal control. Therefore, studies on the secretome of OSCC cell lines may be conducted in order to study the role of ATX in more detail. As ATX is a secreted protein, is it elevated in the secretome? Also, does the secretome of OSCC cells induced ATX expression in the non-malignant cells of the stroma, such as cancer-associated fibroblasts and macrophages.

Secondly, the LPAR1/3 inhibitor that was used in the migration, invasion and radioresistance assays was Ki16425. It was the only inhibitor that was commercially available to us at the time of study. It will be very informative if all the assays are conducted with any available inhibitor which is specific for LPAR1 or LPAR3

individually to further confirm the results from this study. This chapter also investigated the importance of LPAR3 in the LPA-induced migration, invasion and radioresistance assays *in vitro*. The next step would be to examine the functional significance of LPAR3 *in vivo* where cells with stable LPAR3 knockdown could be tested in animal models of oral cancer.

Individual inhibitors targeting any of the major pathways of EGFR, LPAR and COX-2 could have the potential of becoming therapeutics in the treatment of OSCC. However, since there is a possibility of a positive feedback loop (as discussed in section 7.3), that could be formed between these three pathways, a combination of inhibitors would be able to control this loop. Investigating the combination of inhibitors between these three pathways would hopefully overcome or reduce more of the functional expression involved in oral carcinogenesis.

Thirdly, as discussed in section 7.4.1, the most abundant LPA species found in normal plasma samples was LPA 18:2e344. LPA 18:1 is the most commonly used LPA species in the laboratory for signalling studies (Mirendil et al., 2013). LPA 18:1 was also used in all assays in this study. It would be interesting to test other major LPA species using the same assay especially LPA 16:0 since it was found to be the most abundant in OSCC plasma samples. Another important point in this section is to analyse using PCA with other cancerous samples. This exploratory analysis will enhance the potential of using LPA as a potential biomarker in cancer. The next step is generating a predictive model called PLSDA which may be used to differentiate aggressiveness within the early or advanced cancerous groups.

# CHAPTER 8:

# CONCLUSIONS

The present study reveals that the ATX-LPA signalling axis is aberrant in OSCC. This is important because major components of the axis, such as the receptors, represent potential therapeutic targets for this disease. This study has shown that upregulation of LPAR3 promotes migration, invasion and radiation resistance in OSCC. In the future, the development of specific LPAR3 antagonists might be helpful to inhibiting these malignant phenotypes to prevent the progression and metastasis of OSCC. Data from the present study also showed that COX-2 played an important role as a downstream mediators of this signalling axis particularly via LPAR3. Therapeutics targeting the receptors or downstream mediators alone is likely to be less successful compared to targeting both components simultaneously. Therefore, by having inhibitors targeting the receptors together with the inhibitors of downstream mediators would likely be more successful in the strategy to combat this disease. In addition to this, LPA profiles were shown to be altered in OSCC for the first time. Understanding the biological difference between these LPA species in terms of binding to individual LPARs and elucidating if they have different biological roles in cancer and other diseases would be a major advance for the lysophospholipid research field.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

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| 1. | Mariati Abdul Rahman. Investigation Into The Roles Of Lysophosphatidic Acid (LPA) In The Development And Progression Of Oral Cancer, And Inhibitors Of LPA As Novel Therapeutic Targets. Presented at PhD Proposal Defence seminar (2013), Faculty of Dentistry, University of Malaya, Kuala Lumpur. |
| 2. | Mariati Abdul Rahman. Stop migration. Presented at Three Minute Thesis Competition (2015) (Faculty level), University of Malaya, Kuala Lumpur. |
| 3. | Mariati Abdul Rahman. Investigation into the roles of LPA in the pathogenesis of HNSCC. Presented at PhD Candidature Defence seminar (2016), Faculty of Dentistry , University of Malaya, Kuala Lumpur |
| 4. | Mariati Abdul Rahman. Investigation into the roles of LPA in the pathogenesis of OSCC. Presented at PhD Thesis submission Defence seminar 2019, Faculty of Dentistry , University of Malaya, Kuala Lumpur |
| 5. | M Abdul Rahman, SP Johnson, LF Yap and IC Paterson. Dysregulation of Lysophosphatidic Acid (LPA) Signalling Stimulates Cell Migration And Invasion In Oral Squamous Cell Carcinoma**.** Poster presentation at Controlling Cancer Summit, the O2, London 17$^{th}$-19$^{h}$ June 2016. |