Investigating the insulin-like growth factor axis in head and neck cancer

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Contents

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest cancer in the UK. Despite recent therapeutic developments, survival rates remain poor, particularly in advanced cancer and Human Papilloma Virus (HPV) negative disease. Novel treatments approaches are therefore urgently required. The type-1 insulin-like growth factor receptor (IGF-1R) regulates cellular growth and survival and is over-expressed in a range of cancer types. Other groups reported that inhibition of IGF-1R reduces HNSCC cell survival and sensitises to ionising radiation, but a clinical trial of IGF-1R inhibition as monotherapy was inactive in unselected palliative patients with HNSCC. These data suggest that predictive biomarkers for response to IGF-1R inhibition are required.

The aims of this project were threefold. The first objective was to define factors associated with morbidity and mortality in patients with oropharyngeal cancer (OPSCC) treated with primary surgery alone or with adjuvant chemoradiotherapy. The five-year overall and disease specific survival rates were 68% and 78% respectively. In line with previous data, HPV negative status, current smoking status, high tumour T stage and the presence of perineural spread of tumour or lymphovascular invasion were associated with adverse survival outcomes. In surviving patients, quality of life outcomes were evaluated using the University of Washington Quality of Life score and functional outcomes were assessed with the MD Anderson Dysphagia Inventory. Increasing age, higher tumour T stage, lip-splitting mandibulotomy and free flap reconstruction were associated with reduced quality of life outcome scores following multivariate analysis.

The second aim was to assess the significance of IGF-1R expression in HNSCC and test for correlates with clinico-pathological variables. Immunostaining of cores from

346 primary HNSCCs showed that IGF-1R expression was higher in tumour tissue than matched benign epithelium. High IGF-1R was significantly associated with reduced overall and disease specific survival, HPV negative status and high tumour T stage, although was not an independent predictor of survival in multivariate analysis.

The final aim was to test the utility of IGF-1R inhibition in HNSCC cell lines as monotherapy and in combination with established treatments, aiming to identify predictive biomarkers for resistance to IGF-1R inhibition. In a panel of 6 HNSCC cell lines, the IGF-1R inhibitor BMS-754807 reduced IGF-1R, AKT and ERK phosphorylation in a dose dependent manner. IGF-1R inhibition with BMS-754807 reduced cell survival and sensitised cells to ionising radiation in clonogenic assay, although the magnitude of this effect varied between cell lines. Combination of BMS-754807 with the EGFR inhibitor Gefitinib caused supra-additive reduction in cell survival. Correlation analysis showed a trend towards an association between high levels of phosphorylated AKT and resistance to BMS-754807 monotherapy. To test the hypothesis that RAS signalling conferred resistance to IGF-1R inhibition, cells were infected with retroviral constructs encoding wild-type or mutant activated HRAS. Cells expressing mutant HRAS were more resistant to BMS-754807 than empty vector or wild-type HRAS infected controls, suggesting that HRAS mutation status may represent a biomarker of resistance to IGF-1R inhibition in HSNCC. Taken together, the results from this project highlight the significance of IGF-1R biology in HNSCC, and form the basis for further *in-vivo* and clinical research.

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List of Figures

List of Tables

Abbreviations

AKT	Protein Kinase B
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CRT	Chemoradiotherapy
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dose-modifying factor
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal related kinase
EV	Empty vector
FCS	Foetal calf serum
GI_{50}	Concentration that causes 50% growth inhibition
Gy	Gray
HNSCC	Head and neck squamous cell carcinoma
HPV	Human Papilloma Virus
HRAS	Harvey rat sarcoma viral oncogene homolog
IGF-1/2	Insulin-like growth factor 1/2
IGF-1R	Insulin-like growth factor receptor type 1
IHC	Immunohistochemistry
IR	Insulin Receptor
ISH	In-situ hybridisation
MDADI	MD Anderson Dysphagia Inventory
OPSCC	Oropharyngeal squamous cell carcinoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidyl inositol-3 kinase
PTEN	Phosphatase and tensin homolog
P53	Protein 53
Rb	Retinoblastoma
SF_{50}	Concentration at which cell survival is 50% that of solvent control
SF_{75}	Concentration at which cell survival is 75% that of solvent control
si-RNA	Short interfering RNA
TBS-T	Tris buffered saline + 0.1% Tween 20
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
UICC	Union for International Cancer Control
UWQoL	University of Washington Quality of Life (questionnaire)
WT	Wild Type

1 Introduction

1.1 Head and neck cancer

The term head and neck cancer comprises a range of tumour types affecting different sites within the head and neck. Over 90% of head and neck malignancies are squamous cell carcinomas (HNSCC) arising from the upper aerodigestive tract (trachea, larynx, hypopharynx, oropharynx, oral cavity, lip, nose and paranasal sinuses). HNSCC causes significant morbidity and mortality for affected patients, and presents clinicians with considerable therapeutic challenges. The proximity of tumours to vital structures means that treatment often causes serious long-term side effects, and despite the radical nature of treatment a significant number of patients go on to develop recurrent disease or second primary tumours. For this reason, novel therapeutic approaches are urgently required.

1.1.1 Epidemiology

HNSCC is the sixth commonest cancer by incidence, with 650,000 new cases diagnosed per year worldwide and 350,000 HNSCC related deaths (Parkin et al., 2005). Significant geographical variation has been reported, with the highest incidence seen in south-central Asia and central and southern Europe, and the lowest incidence in South America (Parkin et al., 2005). The peak incidence of the disease occurs between the ages of 55-64, and there is a strong male preponderance (Howlader N, 2013). Although HNSCC may affect any area of the upper aerodigestive tract, the commonest sub-sites affected are the oral cavity, the oropharynx and the larynx (Healthcare Quality Improvement Panel, Eighth annual head and neck cancer audit, 2012). Despite a slight reduction in the incidence of HNSCC as a whole over the last four decades, the incidence of oropharyngeal

cancer has increased significantly over the same period (Chaturvedi et al., 2011). This may relate to changes in exposure to well established risk factors.

1.1.2 Risk factors

Several large epidemiological studies have identified smoking and alcohol as risk factors for the development of HNSCC (Blot et al., 1988, Vineis et al., 2004). The risk of developing HNSCC is though to increase in an exposure-dependent manner with both smoking and alcohol, while the combination of both risk factors together has a synergistic, rather than additive effect: For example, patients with a >40 pack year smoking history have an odds ratio of 7.4 for the development of HNSCC, while patients who drink >30 alcoholic drinks per week have an odds ratio of 5.8. However, in patients who have a >40 pack year smoking history *and* drink >30 alcoholic drinks per week the odds ratio for the development of HNSCC is 37.7 (Blot et al., 1988). These risk factors may exert a predilection for certain sub-sites within the head and neck. Alcohol consumption is associated with a high risk of developing hypopharyngeal cancer, while smoking is strongly associated with laryngeal cancer (Menvielle et al., 2004, Tuyns et al., 1988). Evidence also suggests that the particular type of alcoholic beverage consumed influences cancer risk; the consumption of spirits and beer are associated with higher risk of developing HNSCC than wine (Blot et al., 1988). The influence of smoking and alcohol on HNSCC may extend beyond the risk of developing primary HNSCC. Continued smoking and alcohol consumption following treatment for HNSCC is associated with prolonged gastrostomy tube dependence and a significantly higher risk of developing a second head and neck malignancy (O'Shea et al., 2015, Schwartz et al., 1994). Over the last twenty years however, the prevalence of smoking has fallen in Europe and North America which may in turn relate to the slight reduction in the incidence of HNSCC seen over the same period (Lifestyle statistics team, 2014, Office on Smoking and Health, 2014).

While smoking rates have fallen in western countries, they remain high in areas of south-central Asia (Rani et al., 2003). In addition to the high prevalence of smoking in these areas, the chewing of betel nuts is also widespread. This has been identified as an independent risk factor for the development of oral cancer (Merchant and Pitiphat, 2015). The combination of these factors may influence the high incidence of HNSCC observed in this area.

Another major risk factor for HNSCC is Human Papilloma Virus (HPV) infection. The association between HPV infection and HNSCC was first identified in the 1980s, and since then, it has become widely recognised as a risk factor for oropharyngeal squamous cell carcinoma (Snijders et al., 1992, Chaturvedi et al., 2011, Schwartz et al., 1998). HPV is an epitheliotropic, double stranded DNA virus, with over 100 different subtypes (Leemans et al., 2011). Infection with the high-risk oncogenic HPV subtypes 16 & 18 results in expression of the viral oncogenes E6 and E7 in infected cells, which leads to inactivation of p53 and Retinoblastoma (Rb) respectively (Leemans et al., 2011). This results in unchecked cell cycle progression from G1 to S phase, and inhibition of p53 mediated apoptosis (Leemans et al., 2011). These cellular mechanisms underlying HPV associated HNSCC differ considerably from those seen in non-HPV associated disease, which is characterised by mutations in p53, CDKN2A, PTEN, PI3K and HRAS (Stransky et al., 2011). The functional significance of these changes is discussed in sections 1.1.3 & 1.2.3. By contrast, HPV positive disease is associated with approximately half the mutation rate seen in HPV negative disease (Stransky et al., 2011).

In addition to its distinct tumour biology, HPV positive HNSCC is markedly different to HPV negative disease in both its epidemiology and outcome. Patients with HPV positive HNSCC tend to be younger than those with HPV negative HNSCC, and after adjustment for age, and other demographic variables have significantly better overall survival rates, regardless of treatment modality (Ang et al., 2010, Licitra et al., 2006).

Recent evidence suggests that the incidence of HPV associated oropharyngeal cancer has increased significantly over the last 30 years in Europe and North America (Schache et al., 2011, Chaturvedi et al., 2011).

Given the prognostic significance of HPV in HNSCC, determination of HPV status is an important part of the diagnostic process. A variety of different methods have been employed. The gold standard means of detecting biologically relevant HPV infection is thought to be quantitative PCR (qPCR) for viral mRNA performed on fresh-frozen tumour specimens (Schache et al., 2011). This distinguishes biologically meaningful HPV infection by detecting HPV16 E6 gene expression, indicating integration of the viral DNA into the host genome. In routine clinical practice, however, the utility of RNA and DNA qPCR is limited, because it is ineffective in formalin fixed tissue (Leemans et al., 2011).

P16 immunohistochemistry (IHC) provides another means of determining HPV status in HNSCC tissue. The inactivation of Rb by the viral oncogene E7 causes activation of E2F transcription factors, which results in an accumulation of the regulatory protein P16, in a negative feedback loop (Leemans et al., 2011). P16 is readily detectable by IHC in formalin-fixed HNSCC tissue, and provides an inexpensive surrogate measure of HPV infection, which is widely used in clinical practice. P16 immunostaining in HNSCC tissue has high sensitivity for HPV infection (94%), but a lower specificity (82%), when compared to mRNA qPCR, meaning that P16 IHC tends to overestimate the number of HPV positive cases (Schache et al., 2011).

In-situ hybridisation (ISH) for HPV DNA has also been used for determination of HPV status. Like P16 IHC, this can be performed in formalin fixed tissue. Used in isolation, HPV DNA ISH offers lower sensitivity (88%) than P16 IHC, but higher specificity (88%) (Schache et al., 2011). The difficulties associated with accurately identifying HPV status in HNSCC tissue when using a single testing modality have led many

authors to advocate dual modality testing (Schache et al., 2011, Westra, 2009). Westra et al suggest dual testing with P16 IHC and HPV DNA ISH to reduce the number of false positive results from using P16 IHC alone, increasing test specificity (Westra, 2009).

1.1.3 The biology and genetics of HNSCC

Cancer is characterised by the transformation of normal cells into malignant cells, which are capable of invasion and distant metastasis (Hanahan and Weinberg, 2000). This transformation is a multi-stage process, which is driven by mutations in oncogenes - which cause cells to develop a malignant phenotype when mutated (Adamson, 1987), and tumour suppressor genes – which protect cells against malignant transformation, and where loss of function is associated with the development of cancer (Sherr, 2004).

Mutations in oncogenes are referred to as 'dominant', because a mutation in one allele is sufficient to induce malignant transformation. By contrast, tumour suppressor genes are usually recessive, meaning that both alleles of a particular gene must be affected for a malignant phenotype to develop (the two-hit hypothesis) (Sherr, 2004). Examples of established oncogenes in HNSCC include *EGFR, HRAS, PIK3CA* & *MET*, while *p53*, *PTEN* and *Rb* have been identified as tumour suppressor genes (Leemans et al., 2011).

In 2000, Hanahan and Weinberg published a seminal paper identifying six 'hallmarks of cancer', which lead to a cell developing a malignant phenotype: a) self-sufficiency in growth signals, b) insensitivity to antigrowth signals, c) evasion of apoptosis, d) limitless replicative potential, e) sustained angiogenesis and f) tissue invasion and metastasis (Hanahan and Weinberg, 2000). They then went on to identify two further features of cancer cells, which contribute to the malignant phenotype; the ability to

reprogram energy metabolism and evade immune destruction (Hanahan and Weinberg, 2011). The authors suggest that genomic instability in established oncogenes and tumour suppressor genes, as well as a local inflammatory response underlies these cellular processes. The latter is likely to contribute to the recruitment of normal cells to form a 'tumour microenvironment', which allows cancer cells to develop some of the 'hallmark' traits previously described (Hanahan and Weinberg, 2011).

The pathogenesis of HNSCC is complex, and is likely to relate to a number of genetic and epigenetic alterations involving inactivation of tumour suppressor genes and activation of oncogenes (Argiris et al., 2008). In addition, recent data from two large studies indicate that there is considerable genetic and biological heterogeneity between tumours (Lawrence, 2015, Stransky et al., 2011).

In 2011 Stransky and colleagues published data from whole exome sequencing of 74 HNSCCs and paired normal tissue. This study indicated that *TP53, CDKN2A, PTEN, PIK3CA,* and *HRAS* genes are frequently mutated in HNSCC. Of the 74 cases included in this study, 11 were HPV positive. These tumours demonstrated a different genetic profile from HPV negative tumours, with fewer Guanine to Thymine transversions, and a mutation rate half that of HPV negative disease (Stransky et al., 2011). A subsequent study by The Cancer Genome Atlas Network demonstrated that HPV positive HNSCC is characterised by *PIK3CA* mutations, while in HPV negative disease *TP53* mutations predominate, with mutations or deletions occurring in up to 62% of cases (Lawrence, 2015). These findings underline the biological differences between HPV positive and HPV negative HNSCC, and may influence the differences in their response to treatment and prognosis.

1.1.4 Prognostic indicators in HNSCC

Although some significant therapeutic advances have occurred over the last 30 years, survival rates in HNSCC have only improved modestly over the same period (Howlader N, 2013). The majority of patients have loco-regionally advanced disease at presentation, often meaning that radical treatment is required to effect a cure (Argiris et al., 2008). Despite extensive and often disfiguring treatment, 50% of patients will develop either a local or distant relapse of disease (Argiris et al., 2008). In addition, the risk of developing a second primary (metachronous) head and neck tumour is in the region of 3-5% per year (Khuri et al., 2006). Several factors are known to influence survival outcomes in HNSCC, and these may relate to both patient and tumour characteristics.

As previously discussed, heavy cigarette smoking and high levels of alcohol intake are associated with increased risk of recurrence and reduced overall survival following treatment for HNSCC. Other patient factors, which may influence prognosis, include poor patient performance status and increased age which are both associated with reduced overall and progression free survival (Ang et al., 2010). The presence of pre-treatment anaemia (haemoglobin <13.5g/dL in men and <12.5g/dL in women) is also predictive of reduced overall survival in HNSCC (Ang et al., 2010, Denis et al., 2004). This may relate to the prominent role of radiotherapy in treating HNSCC; radiation causes less DNA damage in low oxygen environments (Bentzen et al., 2015). Another patient-related factor that affects survival outcome in HNSCC is co-morbidity. In a recent study of over 9000 patients by the Danish DAHANCA group, the presence of significant co-morbidity was associated with reduced overall survival in HNSCC patients treated with radiotherapy (Boje et al., 2014). In that study, comorbidities were evaluated individually and five (cardiac failure, cerebrovascular disease, chronic respiratory disease, gastric ulcer disease, hepatic disease and diabetes) were found to be strongly associated with adverse survival at 5-years.

Several of these co-morbidities share risk factors with HNSCC (smoking & alcohol), and are thus common in patients being treated for HNSCC, occurring in up to 36% of cases (Boje et al., 2013).

Alongside patient co-morbidity, pre-treatment symptom severity also predicts survival in HNSCC. In a recent Norwegian study, patients were asked to complete health related quality of life inventories before treatment. The authors used the QLQ-C30 and the QLQ-HN35 questionnaires, which contain symptom indices for common symptoms in HNSCC including pain, nausea/vomiting, breathing difficulty, fatigue, anorexia, constipation and diarrhea. They found that patients with the highest pre-treatment symptom scores (indicating more severe symptoms) had significantly lower overall survival than those with the lowest symptom scores (Osthus et al., 2013). The impact of both co-morbidity and symptoms on survival in HNSCC is underlined by a study by Pugliano et al. Following multivariate analysis, they showed that symptom severity and co-morbidity more accurately predicted disease specific and overall survival than TNM stage (Pugliano et al., 1999).

A number of tumour factors have also been shown to influence prognosis in HNSCC. High tumour T stage (UICC TNM staging classification system for HNSCC, 7th Edition), indicating larger tumour size, is associated with increased risk of local recurrence and nodal metastasis as well as reduced overall survival in HNSCC (Woolgar, 2006, Woolgar et al., 1999, Ang et al., 2010). Although T stage is a prognostic indicator in HNSCC, recent evidence suggests that tumour thickness may be of greater prognostic value for some HNSCC sub-sites (O'Charoenrat et al., 2003). Indeed for oral SCC, multivariate analysis suggests that tumour thickness, not tumour T stage is independently predictive of survival outcome (Gonzalez-Moles et al., 2002).

The presence of nodal metastases is another significant prognosticator in HNSCC (Ang et al., 2010). In a retrospective study of 3887 patients with HNSCC of the oral cavity, oropharynx, larynx and hypopharynx, node positive disease (N1-3, UICC TNM staging classification system for HNSCC, 7th Edition), was associated with significantly worse disease specific survival than node negative (N0) disease (Layland et al., 2005, Janot et al., 1996). Recent evidence suggests that other features of nodal involvement may also be important in determining outcome in HNSCC. The presence of bilateral nodal metastases is thought to confer a particularly poor prognosis (Kowalski et al., 2000). This leads to one of the main criticisms of the TNM staging system; that increasing TNM stage does not directly relate to prognosis: N2c HNSCC (bilateral nodal involvement) carries a poorer prognosis than N3 disease (single ipsilateral node >6cm) (Kowalski et al., 2000). The association between advanced disease and adverse outcome is well established, but certain sub-sites within the head and neck are also associated with particularly poor survival in HNSCC. Hypopharyngeal tumours in particular are associated with significantly worse overall and disease specific survival than oropharyngeal, laryngeal and oral cavity tumours (Mehanna et al., 2010b).

In addition to key macroscopic tumour variables including stage and site of disease, certain pathological variables have also been identified which are associated with reduced survival in HNSCC. A large meta-analysis of 9 studies containing data from 2573 patients showed that the presence of extracapsular spread (ECS), which was present in 62% of nodal metastases, was associated with a significantly increased risk of death at 5 years. The 5-year overall survival rates for those with ECS were 30.7%, compared to 58.1% for patients with no ECS (Dunne et al., 2006).

The degree of tumour differentiation (histological grade) has been shown to affect both the risk of metastasis and outcome in HNSCC. Poorly differentiated tumours are associated with a higher rate of local lymph node metastases, and isolated distant

metastases (Janot et al., 1996, Magnano et al., 1999, Lim et al., 2010). An independent association has also been identified between the degree of differentiation and survival, with poorly differentiated tumours associated with reduced overall survival at 3 years (Woolgar et al., 1999, Goto et al., 2013).

Perineural invasion (PNI) of small nerves in the vicinity of the primary tumour is another clinico-pathological variable associated with adverse outcome. In a study of 142 patients with HNSCC, Fagan and colleagues showed that PNI was associated with a significantly increased risk of local recurrence and lower disease specific survival (Fagan et al., 1998). The presence of lymphovascular invasion (LVI) in the primary tumour has been shown to exert a similar effect. LVI is associated with higher risk of nodal metastasis, increased risk of local recurrence and reduced overall survival (Woolgar et al., 1999, Woolgar, 2006).

Although a major risk factor for the development of HNSCC, HPV positive status is also an indicator of favourable outcome. In a large study of 721 patients with oropharyngeal cancer, Ang and colleagues demonstrated that patients with HPV positive HNSCC (identified by ISH for HPV DNA) had significantly lower risk of death at three years than HPV negative patients (Relative Risk 0.41, 95% Confidence Interval 0.29 to 0.57, $p<0.001$) (Ang et al., 2010). Furthermore, following recursive-partitioning analysis, HPV status was found to be the most significant determinant of overall survival in HNSCC. The authors went on to stratify patients into low, medium and high risk groups based upon HPV status, smoking history, tumour T stage and N stage. They found that low risk patients (HPV positive, < 10 pack year smoking history, nodal status N0-N2a) had an estimated 3-year overall survival rate of 93%, compared to intermediate risk patients (HPV negative, < 10 pack year smoking history, tumour T stage T1-T3) (HR 3.54, 95% CI 1.91 to 6.57) (Ang et al., 2010). The favourable survival outcomes in HPV positive HNSCC have been attributed to the increased chemo-radiosensitivity of HPV positive tumours, however, Licitra and

colleagues have demonstrated that patients with HPV positive disease have improved survival outcomes, regardless of treatment modality (Licitra et al., 2006, Rieckmann et al., 2013).

The Epidermal Growth Factor (EGF) axis is known to play a major role in HNSCC biology. EGF ligands bind to the EGF receptor (EGFR), a trans-membrane tyrosine kinase receptor and member of the HER/ErbB family. This results in receptor hetero-dimerisation and tyrosine kinase activation. EGFR activation induces a signalling cascade, which results in cellular proliferation, angiogenesis and invasion (Baba et al., 2012). EGFR is overexpressed in up to 90% of HNSCC, and recent evidence suggests that high EGFR expression may be associated with reduced overall survival in HNSCC (Lothaire et al., 2006, Zhu et al., 2013, Keren et al., 2014). Hama et al provide evidence that EGFR pathway activity is associated with adverse outcome in HNSCC, independent of EGFR expression levels (Hama et al., 2009). In a cohort study of 82 treatment naïve patients with HNSCC, the authors used western blotting of tumour lysate to demonstrate that high levels of EGFR phosphorylation were associated with shorter progression free survival than those with low levels of EGFR phosphorylation (Hama et al., 2009). The significant role of EGFR in HNSCC has led to it becoming an established therapeutic target in this context (Bonner et al., 2010).

1.1.5 Treatment

The principal treatments employed in the management of HNSCC are surgery, radiotherapy and chemotherapy. These may be used both in a curative and a palliative setting. The aim of curative treatment is to obtain loco-regional and distant control of disease and achieve disease free survival, while palliative treatment aims to achieve symptom control and extend survival duration.

Treatment decisions in HNSCC are complex and involve a multi-disciplinary approach, with input from oncological surgeons, plastic and reconstructive surgeons, clinical oncologists, radiologists, histopathologists, dentists, dieticians, speech and language therapists and specialist nurses. Individual treatment decisions are based upon careful consideration of factors relating to both the patient and the characteristics of the particular tumour.

The treatment of early stage HNSCC often involves single modality therapy, either radiotherapy or surgery. In many cases there is clinical equipoise as to which treatment offers the greatest survival benefit due to the lack of clinical trials directly comparing different treatment modalities. The results from separate cohort studies, however suggest that in many cases, results are similar. In early laryngeal cancer for example (T1-2, N0), five-year overall survival rates range from 89-92% for patients treated with radiotherapy and 76-97% for patients treated with primary surgery (Lim et al., 2015, Tong et al., 2012, Moreau, 2000, Ambrosch et al., 1998).

With advancing tumour stage, more radical treatment is required to achieve a cure. Single modality therapy (surgery or radiotherapy) is associated with adverse survival outcome, therefore multimodal treatment is usually employed (Bhalavat et al., 2003, Denis et al., 2004). As the scope of treatment escalates, so too does the morbidity for the patient, which needs to be taken into consideration when making treatment decisions. In the management of locally advanced HNSCC, the three main treatment modalities are a) surgery followed by (chemo)radiotherapy, b) radical cisplatin-based chemoradiotherapy (CRT), c) induction chemotherapy followed by either radiotherapy (RT), chemoradiotherapy or surgery (Seiwert and Cohen, 2005).

In tumours displaying adverse features (positive excision margins, ECS) the addition of adjuvant radiotherapy to primary surgery leads to improved local control and survival (Huang et al., 1992). It appears, however, that the benefit of RT in this

setting may be augmented by the addition of chemotherapy as a radiosensitising agent. In 2004 Cooper and Bernier published the results of two separate large Phase III trials indicating that surgery followed by adjuvant CRT conferred a progression free survival benefit of approximately 10% at 5 years compared to surgery and adjuvant RT (Cooper et al., 2004, Bernier et al., 2004). This survival benefit does however, come at a cost of significantly increased acute treatment toxicity. More recently, the long-term benefits of postoperative CRT over RT have been called into question. The updated outcome data from RTOG 9501 (Cooper et al. 2004) suggest that there is no difference in disease specific or overall survival at 10 years with postoperative CRT compared to RT, except in high risk sub-groups (Cooper et al., 2012).

Although primary surgery with adjuvant RT or CRT remains an accepted treatment modality in advanced HNSCC, it often results in significant functional impairment. This is particularly true in patients with laryngeal or hypopharyngeal tumours, which require surgical excision of the larynx (laryngectomy) (Shah and Tollefsen, 1974). This has led to efforts to achieve organ preservation by using primary RT or CRT in the management of advanced HNSCC. The trend towards organ preservation was initiated by the VA Laryngeal Cancer Study Group, who performed a prospective, randomized trial in patients with advanced laryngeal cancer. Patients were randomized to receive induction chemotherapy with cisplatin and 5-fluorouracil, followed by radiotherapy or primary surgery followed by radiotherapy. Following treatment, the two-year survival estimates for both groups were identical (2 year OS: 68%, 95% CI 60-76%), and resulted in a 64% organ preservation rate in the CRT group (The Department of Veterans Affairs Laryngeal Cancer Study Group, (1991)). These results suggested that organ preservation is achievable in almost two thirds of patients with advanced laryngeal cancer with equivalent survival rates to primary surgical treatment. Since the publication of the VA trial however, evidence has

emerged that concurrent CRT with cisplatin and 5-fluorouracil offers higher rates of loco-regional control and organ preservation than induction CT followed by RT, and it has become the standard of care for patients with advanced HNSCC (Forastiere et al., 2003, Seiwert and Cohen, 2005).

Similar to the long-term analysis of the RTOG 9501 trial described above, examination of the 10-year results of the RTOG 91 11 trial showed no survival benefit of cisplatin/5-fluorouracil CRT over RT alone for advanced laryngeal cancer (Forastiere et al., 2013). The authors showed that treatment with CRT was superior to radiotherapy alone in achieving laryngeal preservation, but had no effect on disease-specific or overall survival. In advanced laryngeal cancer, organ preservation is a highly desirable outcome, and in the GORTEC 2000-01 trial, Pointreau and colleagues showed that even higher rates of laryngeal preservation may be achievable by treatment escalation (Pointreau et al., 2009). In this study, the authors demonstrated that induction chemotherapy with Docetaxal (D), Cisplatin (P) and 5-Fluorouracil (F) followed by DPF chemoradiotherapy resulted in a higher rate of larynx preservation than an equivalent regimen using PF chemotherapy (larynx preservation: DPF = 70.3%, PF=57.5%, p=0.03). Again however, no difference in overall survival between the two groups was seen (overall survival: DPF = 60%, PF=60%, p=0.57). Although late toxicities were comparable between both arms of this study, data on long-term laryngeal function are not presented. The use of 'Laryngectomy-free survival' in this context, may therefore fail to address the increase in morbidity associated with treatment escalation (Denis et al., 2004). This is an important consideration given the comparable long-term survival of patients with advanced laryngeal cancer treated with either RT or CRT (Forastiere et al., 2013).

With comparable survival outcomes and a high chance of organ preservation, the benefits offered by primary CRT for laryngeal/hypopharyngeal cancer are clear.

However, the superiority of CRT is less clear for tumours affecting other sub-sites in the head and neck, where organ preservation is not a treatment goal, such as in oropharyngeal cancer (OPSCC). In the era of HPV positive OPSCC, treatment selection has become particularly important for several reasons. Firstly, evidence from translational and clinical studies suggests that HPV positive OPSCC is more sensitive to treatment than HPV negative disease (Rieckmann et al., 2013, Licitra et al., 2006). This raises the possibility of de-escalating treatment in this group of patients; aiming to achieve similar cure rates with lower treatment morbidity (Mirghani et al., 2015). Secondly, the younger age and improved survival outcomes of patients with HPV positive OPSCC mean that any sequelae of treatment are likely to impact upon patient quality of life for a considerable period of time. There have been no randomised controlled trials comparing surgery with adjuvant CRT to primary CRT in patients with oropharyngeal cancer, and treatment choices are made on the basis of tumour resectability, local expertise and patient preference (Seiwert and Cohen, 2005). Over the last 20 years, there has been a trend towards non-operative management of OPSCC, however, long-term survival and functional outcomes from different treatment modalities in the HPV era are yet to be defined (Chen et al., 2013).

1.1.6 Recent treatment advances

Over the last decade some significant advances have occurred in the management of patients with HNSCC. The widespread introduction of Intensity Modulated Radiotherapy (IMRT) is one such example. IMRT delivers conformal radiotherapy to the tumour with significantly lower doses to surrounding tissues. This results in significantly less xerostomia (dry mouth) following treatment, without compromising survival (Nutting et al., 2011, Gupta et al., 2012). Advancements have also been

seen in the field of head and neck cancer surgery. The use of trans-oral laser surgery is well established in the management of HNSCC, but its use is limited in cases where access is difficult, particularly in tumours of the oropharynx. Trans-oral robotic surgery (TORS) offers a technical solution to this problem, allowing for greater access, three dimensional visualisation and 360-degree manipulation of tissue in confined areas of the upper aerodigestive tract. Although initial outcomes from TORS are encouraging, long term survival and functional outcomes are not available due to the recent introduction of the technique (Dowthwaite et al., 2012).

As previously described, overexpression of EGFR and high levels of EGFR phosphorylation are associated with adverse prognosis in HNSCC. These findings have rendered EGFR an attractive target in this setting. In a large Phase III randomised trial, Bonner and colleagues showed that the addition of cetuximab (CTX) to RT for patients with locally advanced HNSCC leads to significantly improved survival at 5 years compared to RT alone (Bonner et al., 2010). This survival benefit was in the region of 10% at 5 years (5 year overall survival: CTX + RT = 46%, RT = 36%, p=0.02), which is comparable to that which is achieved with CRT when compared to RT alone (Bernier et al., 2004). These findings led Ang and colleagues to investigate whether the addition of CTX to CRT in locally advanced HNSCC could further augment survival (Ang et al., 2014). The results of this trial showed no significant difference in 30 day mortality, progression free survival, loco-regional failure or three year overall survival between treatment arms. In fact, the combination of CTX with CRT led to more interruptions to radiotherapy and more acute toxicity than CRT alone. There is however evidence that cetuximab increases the efficacy of platinum based chemotherapy delivered in a palliative setting (Vermorken et al., 2008).

Although several significant treatment advances have taken place in the last 10 years, survival rates in HNSCC, particularly in HPV negative disease remain poor.

This makes it a priority to develop novel treatment approaches based on an understanding of tumour biology.

1.2 The Insulin like Growth Factor (IGF) axis

The insulin-like growth factor (IGF) axis is an important regulator of growth and development (Maki, 2010). Recent evidence suggests that it may also play a key role in the development and progression of several common types of cancer including HNSCC (Pollak, 2012). IGF biology is regulated by IGF ligands (IGF-1 and IGF-2), the type 1 IGF receptor (IGF-1R) and type 2 IGF receptor (IGF-2R), as well as several IGF binding proteins (IGFBPs).

1.2.1 IGF ligands and binding proteins

IGF-1 and IGF-2 ligands are small proteins, which are secreted by the liver in response to growth hormone (GH) stimulation (Maki, 2010). IGF-1 and IGF-2 are present at concentrations of 9-35nmol/L and 80-130nmol/L in the serum respectively (Pollak, 2012). Both ligands have similar amino acid sequences and each bind to IGF-1R. In addition, IGF-2 binds to the IGF-2R. IGF-2R however, has no signalling activity but causes internalisation and degradation of IGF-2 ligand (Pavelic et al., 2002).

Both IGF ligands have been shown to play a crucial role in growth and development; mice with null mutations of either IGF-1 or IGF-2 genes suffer from significant growth retardation (Liu et al., 1993). Although both ligands bind to IGF-1R, the receptor has a higher affinity for IGF-1 than IGF-2, and as a result, IGF-1 is though to be responsible for the majority of IGF-1R signalling (Clemmons, 2007). In addition to binding to IGF-1R, IGF-2 also binds to the 'type A' splice variant isoform of the insulin receptor (IR). This is commonly expressed in cancer cells, and is likely to contribute to the prominent role of IGF signalling in cancer as well as the pro-neoplastic effect of hyperinsulinaemia in patients with type-2 diabetes (Belfiore et al., 2009).

The bioavailability of IGF ligands is tightly controlled by a number of IGFBPs. There are six IGFBPs in total, which are synthesised in the liver and interact to regulate serum levels of IGF-1 and IGF-2. Under normal conditions, over 90% of IGF ligand is bound by IGFBPs, which exhibit a higher affinity for ligand than the IGF-1R (Pollak, 2012, Horney et al., 2001). As well as regulating serum IGF levels, evidence has recently emerged suggesting that IGFBPs exert distinct antiproliferative and antineoplastic effects independent of their ability to bind IGF ligand (Jogie-Brahim et al., 2009).

1.2.2 The IGF-1R

The IGF-1R is a tetrameric transmembrane tyrosine kinase receptor, which is encoded by the *IGF-1R* gene, located on chromosome 15. Transcription of the *IGF-1R* gene yields mRNA, which is translated into a 220kDa polypeptide, linked to a 30 amino acid signal peptide. This pro-receptor undergoes glycosylation and then cleavage in the Golgi apparatus, resulting in a 135kDa α polypeptide and 97kDa β polypeptide. The mature IGF-1R constitutes two extracellular α subunits and two transmembrane β subunits, which are linked by disulphide bonds (Adams et al., 2000).

IGF ligands bind to the extracellular α subunit of IGF-1R, which has a 6-8 fold higher affinity for IGF-1 than IGF-2. Other ligands that bind IGF-1R include insulin and interferon-gamma (IFN-γ), although significantly higher concentrations are required to induce receptor activation (Forbes et al., 2002). Ligand binding to IGF-1R causes autophosphorylation of tyrosine residues Y1131, Y1135 and Y1136 on the β subunit of the receptor, which induces a conformational change in the cytoplasmic component. This in turn initiates an intracellular protein kinase cascade (Chitnis et al., 2008).

1.2.3 IGF-1R signalling

Following ligand binding and autophosphorylation of tyrosine residues on the kinase domain of the β subunit of IGF-1R, juxtamembrane tyrosine and serine groups undergo phosphorylation. These represent the binding sites of several important docking proteins including insulin receptor substrate (IRS) 1-4, Src homology and collagen domain protein (SHC) and growth factor receptor bound protein 2 (Grb2) (Chitnis et al., 2008). Recruitment of these docking proteins initiates signalling via two major pathways; the phosphatidylinositol-3-kinase (PI3K) pathway and the RAS/RAF/ mitogen-activated protein kinase (MAPK) pathway (Figure 1) (Chitnis et al., 2008).

Signalling through the PI3K pathway is initiated when IRS recruits and binds to PI3K. This induces phosphorylation of phosphatidyl inositol 3, 4-diphosphate (PIP_2) to create phosphatidyl inositol 3, 4, 5-triphosphate (PIP_3), which activates phosphoinositide-dependent kinase-1 (PDK1). The tumour suppressor phosphatase and tensin homolog (PTEN) reverses the phosphorylation of PIP_3 and is thus an important regulator of the PI3K pathway at this level (Stambolic et al., 1998). Activated PDK1 phosphorylates the threonine 308 residue of protein kinase B (AKT) (Manning and Cantley, 2007). A second phosphorylation site of AKT (serine 473) is activated by mammalian target of rapamycin complex 2 (MTORC2) via an unidentified mechanism arising from IGF-1R activation (Carnero, 2010). Phosphorylated thr308/ser473 AKT activates multiple downstream effectors, which act to promote cell survival, growth and proliferation (Chitnis et al., 2008). One such example is MTOR, which is part of the MTORC1 complex and is activated by AKT to promote translation. This leads to upregulation of the proteins c-Myc and cyclin D1, which are essential regulators of growth and proliferation (Manning and Cantley, 2007).

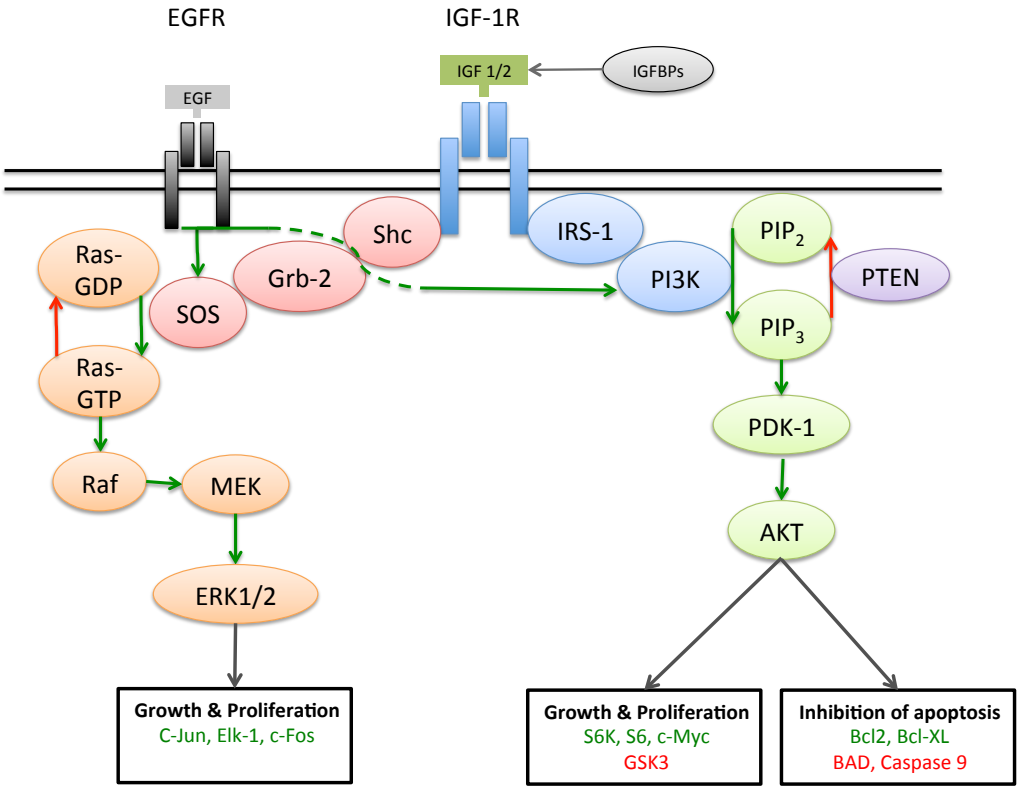


Figure 1: IGF-1R and EGFR signalling

IGF-1R signalling is initiated by IGF1/2 ligand binding to the extracellular α-subunit of the receptor. Phosphorylation of tyrosine kinase residues of the β-subunit of the receptor initiates the binding and phosphorylation of receptor associated binding proteins. Recruitment of IRS-1 and Shc initiates signalling via the PI3K (green) and MAPK (orange) pathways respectively. EGFR activation by ligand also activates the PI3K and MAPK pathways, leading to cellular growth and proliferation via activation of transcription factors (c-Jun, Elk-1 &c-Fos) and inhibition of apoptosis via activation of Bcl2/Bcl-XL and inhibition of BAD/Caspase 9.

The second major pathway associated with IGF-1R signalling is the RAS/RAF/MAPK pathway. When ligand binds to IGF-1R, phosphorylation of serine residues on the β subunit of the receptor allows binding of SHC. This binds Grb2, which in turn recruits and binds to son of sevenless (SOS). SOS activation leads to the removal of a guanosine diphosphate group from RAS (RAS-GDP), which then preferentially binds to guanosine triphosphate (RAS-GTP). RAS-GTP activates RAF kinase, which initiates a protein kinase cascade, phosphorylating mitogen-activated protein kinase 1 and 2 (MEK1/2), which phosphorylate the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Yoon and Seger, 2006). Along with the MAP kinases c-Jun N-terminal kinase (JNK) and p38, which are also activated by RAS-GTP, ERK1/2 promote the transcription of genes which mediate cellular proliferation (Stephen et al., 2014).

Although the PI3K and RAS/RAF/MAPK pathways are the main signalling pathways associated with the IGF-1R, other intracellular signal transduction pathways are also linked to the IGF axis. IGF-1R activation leads to phosphorylation of janus kinase (JAK) that itself phosphorylates signalling transducers and activators of transcription (STATs). These play a key role in regulating growth and proliferation by activating transcription in target genes (Zong et al., 2000).

1.3 IGF-1R in cancer

IGF-1R is widely expressed in most tissues, with the exception of hepatocytes and T lymphocytes and its activity is tightly regulated by the interaction of IGF ligands with IGFBPs (Moschos and Mantzoros, 2002). IGF-1R signalling is required for progression through all stages of the cell cycle and regulates foetal development, skeletal growth and height (Sell et al., 1994, Liu et al., 1993, Yee, 2012). Although it

plays a significant role in development and in the maintenance of the healthy state, aberrant IGF signalling has also been implicated in tumourigenesis.

The prominent role of IGF biology in the progression of cancer was first identified by Lippman and colleagues in 1976, who demonstrated that insulin or IGF ligands caused proliferation of breast cancer cells in-vitro (Osborne et al., 1976). Subsequent work by Baserga et al, showed that IGF-1R also plays an important role in the development of cancer *de-novo*. They showed that IGF-1R is required for the malignant transformation of fibroblasts by most oncogenes including the simian virus 40 (Sell et al., 1993). IGF-1R signalling has since been identified as a key pathway in cancer, stimulating both proliferation and survival pathways, thereby enabling anchorage-independent growth; a feature required for metastasis (Baserga et al., 2003). Further evidence for the role of the IGF axis in cancer comes from xenograft experiments in mice, with disrupted hepatic IGF-1 alleles. These mice have a dwarf phenotype and show reduced growth of murine tumours and reduced susceptibility to mammary and skin carcinogenesis (Moore et al., 2008, Wu et al., 2003, Yakar et al., 1999).

Clinical evidence for the association between IGF and cancer risk comes from epidemiological studies of patients from the Middle East and Ecuador suffering from Laron dwarfism, which is characterised by a mutation in the GH receptor, resulting in short stature and low circulating levels of IGF (Laron, 1993). These patients were followed for over 20 years and found to have significantly lower rates of cancer than unaffected familial controls (Guevara-Aguirre et al., 2011, Steuerman et al., 2011). While low serum levels of IGF are associated with reduced cancer risk, evidence from the general population also suggests that high levels of IGF-1 are associated with a significantly increased risk of developing cancer. A large meta-analysis including 3700 men with prostate cancer and 5200 normal controls showed that high levels of serum IGF-1 are associated with a significantly increased risk of developing

prostate cancer (Roddam et al., 2008). Circulating levels of IGF have also been linked to cancer risk in women. In a large case-control study, Hankinson and colleagues showed a positive correlation between breast cancer risk and serum IGF concentration in premenopausal women (Hankinson et al., 1998). IGF biology may also contribute to the relationship between increasing female height and breast cancer risk identified in the Million Women Study (Green et al., 2011), and to the increased incidence of colorectal cancer in patients with acromegaly (Giovannucci, 2001). To date there is no evidence of correlation between circulating IGF-1 and risk of HNSCC, but there are data to show that high circulating IGF-1 is associated with increased risk of second primary tumours in HNSCC patients (Wu et al., 2004).

Previous work from this laboratory has shown that IGF-1R is over-expressed in a range of tumour types including prostate, bladder and kidney cancers (Turney et al., 2011, Rochester et al., 2007, Yuen et al., 2007). Results from other studies indicate that IGF-1R expression is also increased in other solid tumours including lung cancer and malignant melanoma (Kanter-Lewensohn et al., 1998, Minuto et al., 1986). In the majority of such cases, IGF-1R up-regulation is likely to result from a preceding molecular event such as tumour suppressor gene inactivation, rather than IGF-1R gene amplification, and is not associated with constitutive IGF-1R activation (Werner and Bruchim, 2012, Werner, 2012). Nevertheless, IGF-1R expression has been linked to survival outcome in these tumour types. In a small study of 48 patients with clear cell renal cancer, Parker and colleagues showed that patients with tumours expressing greater than 50% IGF-1R had a four fold increased risk of death at two years, compared to those expressing less than 50% IGF-1R (Parker et al., 2002). Similar findings have been identified in ovarian cancer (Spentzos et al., 2007).

IGF-1R signalling has also been implicated in the response of cancer cells to ionising radiation. In non-small cell lung cancer (NSCLC) cells, irradiation results in IGF-1R activation, which is detectable up to 8 hours after treatment. This is though to exert a

cytoprotective effect by stimulating binding of Ku86 to DNA, inducing DNA double-strand break repair (Cosaceanu et al., 2007). Consistent with these findings, in-vitro data suggest that inhibition of IGF-1R leads to enhanced radiation sensitivity (Cosaceanu et al., 2007, Riesterer et al., 2011b). Conversely, over-expression of IGF-1R is associated with radioresistance in fibroblasts (Turner et al., 1997b, Nakamura et al., 1997). In the clinical setting, high IGF-1R expression in breast cancer tissue is strongly associated with increased risk of early local recurrence within the irradiated field (Turner et al., 1997b). The prominent role of the IGF axis in cancer biology and radiation response has stimulated interest in targeting the IGF-1R in cancer.

1.4 IGF-1R as a therapeutic target

IGF-1R inhibition may be achieved in a variety of ways. The commonest agents used for both *in-vitro* and *in-vivo* studies are either monoclonal antibodies or tyrosine kinase inhibitors (TKIs) (Baserga, 2005). More recently, a newer class of IGF-1R inhibitors has entered preclinical and clinical trials. Ligand antibodies bind IGFs 1 and 2, thereby reducing signaling via IGF-1R and insulin receptor 'A' variant (Friedbichler et al., 2014), however, there are little data on their clinical efficacy.

IGF-1R monoclonal antibodies bind to the extracellular α-subunit of the receptor and cause receptor internalization and degradation, thereby reducing the amount of receptor at the cell surface available for ligand binding (Romanelli et al., 2007). IGF-1R monoclonal antibodies vary in their structure, sub-type and half life, but they are all specific to IGF-1R, and do not bind to the insulin receptor although some have been shown to bind IGF-1Rs in IGF-1R/insulin hybrid receptors (Sachdev et al., 2006). Because of this effect and the existence of endocrine feedback loops, the use of these antibodies is associated with significant hyperglycaemia. Inhibition of pituitary IGF-1Rs is thought to induce negative feedback from the GH-pituitary axis,

resulting in high levels of GH and GH induced insulin resistance. This also results in hepatic IGF-1 secretion, and hence a significant increase in the concentration of serum IGF-1 (Pollak, 2012). Examples of IGF-1R monoclonal antibodies include IMC-A12 (ImClone Systems Inc) (Burtrum et al., 2003), figitumumab (Pfizer) (Cohen et al., 2005), AMG 479 (Amgen) (Beltran et al., 2009) and R1507 (Roche) (Kurzrock et al., 2010).

Tyrosine kinase inhibitors act on the intracellular β-subunit of the IGF-1R, to inhibit downstream signaling through competitive inhibition of ATP binding or inhibition of substrate binding resulting in inhibition of activating phosphorylation (Chitnis et al., 2008). The high degree of sequence homology between IGF-1R and IR (Ullrich et al., 1986) means that TKIs are not selective for the IGF-1R and also cause inhibition of IR and IGF-1R hybrid receptors (King and Wong, 2012). As expected, treatment with IGF-1R TKIs therefore results in hyperglycaemia, however, this effect is limited because of incomplete IR inhibition in the therapeutic dose range (Pollak, 2012). Examples include OSI-906 (OSI Pharmaceuticals) (Jones et al., 2015), BMS-754807 (Bristol-Myers-Squibb) (Carboni et al., 2009) and NVP-ADW742 (Novartis Pharma) (Warshamana-Greene et al., 2005).

Tyrosine kinases play a fundamental role in the modulation of growth factor signalling pathways, which promote cellular survival, growth, angiogenesis and metastasis (Blume-Jensen and Hunter, 2001). They may be either receptor bound (e.g. IGF-1R, EGFR, VEGFR, PDGFR) or non-receptor bound (e.g. BCR-ABL, KIT). The prominent role of tyrosine kinases in tumorigenesis has rendered them an attractive therapeutic target in a range of tumours, and several have been approved for clinical application (Hartmann et al., 2009, Arora and Scholar, 2005). These agents have similar mechanisms of action to the IGF-1R TKIs previously described, causing competitive inhibition of ATP at the catalytic binding site of oncogenic tyrosine kinases.

Although designed to inhibit specific protein tyrosine kinases, TKIs may inhibit other targets including enzymes, ion channels and protein kinases (Smyth and Collins, 2009). This can leads to 'off target' effects, which may increase the cytotoxicity and side-effect profile of TKIs. As previously discussed, IGF-1R TKIs often cause IR inhibition, but at higher doses they may also inhibit other tyrosine kinases including Aurora kinase, B-Raf and EGFR. Inhibition of these other intracellular kinases, however, occurs at doses 100-fold higher than those required to inhibit IGF-1R (Chen and Sharon, 2013). The lack of specificity of TKIs may however, offer an advantage over other targeted therapies such as monoclonal antibodies. Cellular proliferation, invasion, metastasis and angiogenesis are regulated by multiple signalling pathways, and are rarely regulated by a single receptor or tyrosine kinase. The off target effects of TKIs may therefore contribute to their efficacy (Arora and Scholar, 2005).

Several TKIs are used in the clinical setting. Imatinib (Glivec, Novartis) is a tyrosine kinase inhibitor used in the treatment of patients with chronic myeloid leukaemia (CML), who harbour a t(9;22) translocation, known as the Philadelphia chromosome. This results in formation of the BCR-ABL oncogene, which has tyrosine kinase activity, and causes unchecked proliferative activity in haematopoietic cells. Imatinib inhibits tyrosine kinase residues on both ABL and BCR-ABL, and has significantly improved overall survival rates in CML (Smith, 2011). Over 95% of patients with CML harbour the Philadelphia gene, and BCL-ABL tyrosine kinase activity is required for oncogenic transformation (Arora and Scholar, 2005). Affected cells are therefore 'addicted' to this pathway and are uniquely susceptible to inhibition BCL-ABL tyrosine kinase inhibition.

Another example of a tyrosine kinase inhibitor used in the clinical setting is Gefitinib, a selective EGFR inhibitor, licensed for the treatment of non-small cell lung cancer (NSCLC) following failure of first or second line chemotherapy. In a small proportion

of patients with NSCLC Gefitinib leads to a dramatic reduction in tumour volume, however the majority of patients show no response (Kris et al., 2003). Recently, a point mutation in the *EGFR* gene has been identified which predicts sensitivity to Gefitinib, highlighting the need for predictive biomarkers in this setting (Lynch et al., 2004).

The first study to investigate targeting the IGF-1R was published in 1989. This indicated that inhibition of the IGF-1R using a monoclonal antibody reduced cell survival in breast cancer cells *in-vitro* and *in-vivo* (Arteaga et al., 1989). Since then, further studies have demonstrated the utility of IGF-1R inhibition in a range of cancer cell models both as monotherapy and in combination with other treatments. IGF-1R inhibition has been identified as a potent radiosensitising agent by our group and others (Allen et al., 2007, Riesterer et al., 2011a, Turney et al., 2012, Chitnis et al., 2013). IGF-1R inhibition has also been shown to augment the effect of cytotoxic chemotherapy in preclinical studies (Singh et al., 2014, Bitelman et al., 2013). Finally, the significant cross-talk between the IGF-1R and other intracellular signalling pathways in cancer has rendered inhibition of multiple signalling pathways an attractive treatment strategy in preclinical studies. There is evidence for enhanced IGF-1R inhibitor efficacy when used in combination with inhibitors of EGFR, src and MET in HNSCC and other tumour types (Axelrod et al., 2014, Varkaris et al., 2013, Min et al., 2015).

On the basis of encouraging preclinical data, IGF-1R inhibitors entered clinical trials and initial results were promising. A Phase II trial of carboplatin and paclitaxel in combination with the IGF-1R monoclonal antibody figitumumab in patients with non-small cell lung cancer showed a near doubling of the response rate and increased progression free survival in patients treated with figitumumab. Of particular interest was the sub-group of patients with squamous cell carcinoma, who had an 80% response rate to the combination including figitumumab (Karp et al., 2009). Results

from the Phase III trial, however, were disappointing, and it was terminated early after interim analysis suggested that outcomes were better in the chemotherapy alone arm (Yee, 2012).

Similar negative results were obtained in randomized, double blind Phase II trial using the IGF-1R monoclonal antibody ganitumab (Amgen) in combination with endocrine treatment in patients with hormone receptor positive breast cancer. This showed no benefit of IGF-1R inhibition in this group of patients. Specifically, there was no difference in the median progression free survival between the treatment arms, but overall survival was worse in the group treated with ganitumab (Robertson et al., 2013). Data from *in-vitro* studies suggesting a potent effect of dual EGFR and IGF-1R inhibition in NSCLC has not been substantiated in the clinical setting. A Phase II trial of erlotinib (Roche) in combination with the IGF-1R monoclonal antibody R1507 showed no improvement in progression free survival over erlotinib alone (Ramalingam et al., 2011).

Despite these initial negative results, IGF-1R inhibition has shown some promise as a therapeutic agent in the clinical setting. In particular, monoclonal antibodies targeting IGF-1R have demonstrated significant clinical utility in the treatment of soft tissue sarcomas (Schoffski et al., 2013, Olmos et al., 2010). In addition, several of the studies, which reported initial negative results, have subsequently published subgroup analyses that have identified groups of responsive patients (McCaffery et al., 2013, Watkins et al., 2012). These results taken together with the potent anti-tumour effect of IGF-1R inhibition *in-vitro*, suggest that it may have a place in the treatment of other tumour types, particularly if it proves possible to identify biomarkers that indicate responsive patients.

1.5 IGF-1R in HNSCC

In common with other solid tumours, IGF-1R is overexpressed in HSNCC (Barnes et al., 2007, Mountzios et al., 2013). The significance of IGF-1R overexpression, however is unclear, with studies appearing to present conflicting data. In a cohort of 131 patients with HNSCC, Lara and colleagues showed that high levels of IGF-1R expression were associated with reduced locoregional control and survival in a sub-group of patients with advanced disease, but IGF-1R was not prognostic for the study population as a whole (Lara et al., 2011). In another study of 289 patients with laryngeal cancer IGF-1R expression was an independent prognostic indicator following multivariate analysis, and was associated with reduced overall and disease free survival (Mountzios et al., 2013). The relationship between IGF-1R expression and survival in HNSCC is further complicated by data from Matsumoto and colleagues, which suggest that high IGF-1R expression is associated with HPV negative HNSCC (Matsumoto et al., 2014). As a major prognostic indicator in HNSCC, HPV status is an important confounding variable, which was not included in the studies of Lara and Mountzios. The significance of IGF-1R expression in HNSCC is therefore incompletely understood.

The overexpression of IGF-1R in HNSCC, and the potent effect of IGF-1R inhibition in other cell line models, renders the IGF-1R a potentially attractive target in HNSCC. Several authors have investigated the effect of IGF-1R inhibition in HNSCC using cell line and xenograft models. Barnes and colleagues showed that treatment of HNSCC cell lines with the IGF-1R monoclonal antibody IMC-A12 (Imclone) results in cell cycle arrest, and reduces both anchorage dependent and anchorage independent cell growth. They also demonstrated enhanced growth inhibition when IMC-A12 was used in combination with cetuximab in cell line and xenograft models (Barnes et al., 2007). IGF-1R inhibition has been identified as a potent radiosensitising agent in other cancer cell lines, and similar results have been seen in HNSCC cell lines. This

effect was first demonstrated by Riesterer and colleagues, who showed that IMC-A12 increased the radiosensitivity of FaDu and HN5 cell lines in a dose dependent-manner, with dose-modifying factor (ratio of cell survival at specified radiation doses) of 1.2 to 1.8 (Riesterer et al., 2011b).

With preclinical data indicating that IGF-1R inhibition both augments the effect of EGFR inhibition in HNSCC and causes significant radiosensitisation, Raju et al recently sought to determine whether dual IGF-1R and EGFR inhibition could further enhance radiosensitisation in HNSCC. Using IMC-A12 and cetuximab in combination with irradiation *in-vitro* and in a xenograft model, they demonstrated that dual treatment did not increase radiosensitivity of HNSCC (Raju et al., 2014). In this study, marked variation in the response of HNSCC cell lines to IGF-1R inhibition was observed. This finding has also been noted by Riesterer and colleagues (Riesterer et al., 2011b).

The only clinical trial to test IGF-1R inhibition in HNSCC patients was published by the GORTEC (Groupe D'Oncologie Radiotherapie Tete et Cou) group in 2012. In this trial, 17 patients with advanced palliative HNSCC, who had evidence of disease progression following platinum-based chemotherapy were treated with figitumumab monotherapy. The primary outcome measure in this trial was disease control at 6-8 weeks evidenced by a complete or partial response to treatment or by stable disease. At 6-8 weeks after treatment none of the patients showed a response to treatment, and only two displayed stable disease. At 12 weeks all patients had evidence of disease progression. This led the authors to conclude that figitumumab had no significant activity in this group of patients (Schmitz et al., 2012).

Despite the negative results from the GORTEC trial, preclinical data suggest that there may be a role for IGF-1R inhibition in the treatment of patients with HNSCC, when used in combination with existing treatment modalities. In light of the variability

of the response to IGF-1R inhibition in preclinical studies in HNSCC, and in clinical studies in other solid tumours, identification of biomarkers indicating sensitivity to IGF-1R inhibition is a high priority.

1.6 Hypothesis

This project will investigate the role of the IGF-1R axis in HNSCC. The IGF-1R axis exerts an effect on several important intracellular pathways, which mediate cell survival, cell cycle progression and DNA damage response (Baserga et al., 2003, Osborne et al., 1976). In other solid tumours, high levels of IGF-1R expression are associated with adverse survival outcomes (Parker et al., 2002, Spentzos et al., 2007), but in HNSCC the association between IGF-1R expression and survival is uncertain. In the clinical setting, IGF-1R inhibition has shown promise in the treatment of some solid tumours (Asmane et al., 2012a). The role of IGF-1R inhibition in HNSCC, however, remains to be defined. On the basis of existing pre-clinical and clinical data the hypotheses for this project are as follows:

1. IGF-1R expression is associated with survival outcome in HNSCC.

2. IGF-1R inhibition with tyrosine kinase inhibitors reduces survival of HNSCC cells in-vitro, both alone and in combination with other treatment modalities.

3. Components of the IGF-1R axis may act as a biomarker to indicate sensitivity to IGF-1R inhibition in HNSCC cells.

1.7 Objectives of this project

The aims of this project are as follows:

1. To define the functional and survival outcomes in a cohort of patients with oropharyngeal cancer and known HPV status treated with primary surgery.
2. To determine the effect of IGF-1R expression on survival of patients with HNSCC, in the context of known prognostic factors including HPV status.
3. To evaluate the utility of IGF-1R as a therapeutic target in HNSCC, and identify biomarkers which indicate sensitivity to IGF-1R inhibition.

Ultimately, this project aims to explore novel treatment approaches in HNSCC, and provide translational data to test the efficacy of IGF-1R inhibition in this setting, which may be used to structure future clinical trials.

2 Materials and Methods

2.1 Evaluating outcomes in oropharyngeal cancer

2.1.1 Patients

Consecutive patients undergoing primary surgery for oropharyngeal cancer between 1st January 2000 and 31st December 2010 at the John Radcliffe Hospital in Oxford, UK, were identified from the Oxford Head and Neck Cancer database. The John Radcliffe Hospital in Oxford is a tertiary referral centre for patients with head and neck cancer with a referral base of 2,269,772. The department sees 256 new cases of HNSCC per year (O'Moore E, 2013, BAHNO, 2014).

Oropharyngeal cancer was defined as biopsy-proven squamous cell carcinoma arising from the tonsil, tongue base, soft palate and posterior pharyngeal wall. All cases were discussed in the head and neck multidisciplinary meeting comprising surgeons, oncologists, radiologists and pathologists, and treatment carried out in accordance with ENT UK guidance. HPV status was not used to determine treatment modality. Only patients treated with primary surgery, with curative intent were included in this study. Those receiving radiotherapy/chemoradiotherapy as primary treatment modality, those receiving palliative treatment and those who had been previously treated for head and neck cancer were excluded.

Case note review was performed for all patients to confirm database findings. Demographic details, tumour TNM stage (UICC 7th edition), treatment details, pathological findings and survival outcomes were recorded.

2.1.2 Survival outcomes

Overall survival and disease specific survival were determined for patients with known HPV status based upon P16 immunohistochemistry and in-situ hybridisation for HPV DNA (section 2.2.1). Survival duration was defined as the time from primary surgery until death or last clinical contact. Where survival outcome or cause of death was unknown, the patient's General Practitioner was contacted for further information. Survival estimates were generated using Kaplan-Meier analysis (GraphPad PRISM, v6, Graphpad, USA), and associations between survival and clinico-pathological variables identified with a log-rank test (Stata version 11, Texas, USA).

2.1.3 Functional outcomes

Functional outcomes were identified using two validated head and neck cancer specific quality of life questionnaires and gastrostomy tube data. The MD Anderson Dysphagia Inventory (MDADI) and the University of Washington Quality of Life (UWQoL) questionnaire were sent to surviving patients by post, with instructions on completing the questionnaires and pre-paid return envelopes.

The MDADI consists of 20 questions, grouped into global, emotional, physical and functional domains (Chen et al., 2001). Scores for each question range from 0-100, with a score of 100 indicating maximal function. For each patient, mean scores for each domain (global, emotional, physical and functional) were calculated along with the mean total MDADI score (Chen et al., 2001). The UWQoL questionnaire consists of 12 questions, each with a maximum score of 100, which indicates maximal function. For analysis, questions were grouped into either physical or social/emotional domains as previously described (Rogers et al., 2010). The 'swallowing' score was also analysed as an independent outcome measure as

previously reported by Thomas et al. (Thomas et al., 2008). Mean composite scores for the physical and social/emotional domains were calculated for each patient.

Gastrostomy data were obtained from the department of Dietetics database at the Churchill Hospital Oxford. Gastrostomy tube insertion in patients with OPSCC was performed according to local protocols: Patients receiving bilateral radiotherapy to the oropharynx or neck underwent gastrostomy tube insertion prior to starting treatment. Other patients were managed expectantly. Gastrostomy tube data collected included the date of tube insertion, the duration of tube feeding and the date of tube removal.

Outcome data were entered into contingency tables and analysed using Pearson's chi-square test or Fisher's Exact test. Significant associations were entered into multivariate regression analysis. In multivariate regression analysis age, T stage, AJCC stage, resection type, reconstruction type, chemotherapy and time from treatment were analysed as independent variables. Statistics were performed using Stata version 11 (Stata Corporation, Texas, USA). Statistical analyses were performed by Dr Cheng Han (Department of Oncology, Oxford University).

2.2 Immunohistochemistry

2.2.1 Tumour tissue and HPV status determination

Primary HNSCC tissue from 346 patients, obtained from Oxford, Liverpool and Coventry was compiled to form a tissue microarray (TMA). Tumour tissue from Oxford comprised 107 cases of OPSCC treated with primary surgery at the John Radcliffe Hospital between January 2000 and December 2009. Tissue from Liverpool consisted of 176 cases of laryngeal, oropharyngeal, hypopharyngeal or oral HNSCC treated with primary surgery at Liverpool and Aintree University Hospitals between

February 1998 and January 2009. Tissue from Coventry included 63 cases of oropharyngeal or oral HNSCC treated with either primary surgery or radiotherapy ± chemotherapy at the University Hospital of Coventry and Warwick, between May 1999 and October 2011. The TMA was assembled by the Histopathology departments at Birmingham and Liverpool University Hospitals. Prior to inclusion in the TMA primary tumour tissue sections were examined by a Consultant Head and Neck Pathologist to identify suitable areas for cores to be taken. Three 0.6mm cores were taken from each primary tumour, and TMAs were sectioned at a depth of 4μm. Immunohistochemical staining for $P16^{INK4A}$ was performed on sections using the monoclonal antibody MTM-E6H4 (MTM Laboratories, Heidelberg, Germany) (Charfi et al., 2008). In-situ hybridisation (ISH) for HPV DNA, and was performed using Ventana INFORM HPV probes (Ventana Medical Systems Inc. Tucson, US) which detect common high-risk HPV genotypes (including HPV-16) (Guo et al., 2008). P16 immunohistochemistry and ISH for HPV DNA were performed by the histopathology departments at Birmingham and Aintree University Hospitals. A consultant Head and Neck Pathologist interpreted the results according to local protocols. All human tissue samples were used and stored in accordance with the Human Tissue Act, under National Research Ethics study number 07/H0606/120.

2.2.2 IGF-1R immunohistochemistry

IGF-1R immunohistochemistry used a method developed by Aleksic et al (2010). TMA slides were dewaxed in Citroclear (TCS Biosciences Ltd, UK) for 16 minutes and then serially rehydrated in ethanol (100%, 80%, 70%, 50%) for 2 minutes each and distilled water for 5 minutes. Sections underwent antigen retrieval in Tris/EDTA buffer (Trisma base 50nM, EDTA 2mM, pH9) in a decloaking chamber (DC2002, Biocare Medical, USA) at 125°C for 2 minutes, and then 85°C for 10 minutes. Slides

were left to cool, washed three times with phosphate buffered saline (PBS) and then incubated at room temperature with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. After three further washes with PBS, slides were blocked with 5% goat serum/5% Bovine serum albumin (BSA) in PBS for 1 hour. Primary IGF-1R antibody (#9750, Cell Signaling Technology) at 1:50 dilution in 5% goat serum/5% BSA in PBS was applied to tissues and the slides were incubated at 4°C overnight. After washing with PBS three times, secondary rabbit antibody (Rabbit HRP-Polymer, Menarini Diagnostics) was applied to the slides for 20 minutes. Slides were washed 3 times with PBS, and then incubated with DAB substrate (Envision) for 5 minutes at room temperature. Slides were then washed 3 times with distilled water and counter stained with Mayer's haematoxylin (Vector Laboratories, USA) for 5 seconds. After a final wash with distilled water, tissues were dehydrated in serial dilutions of ethanol (50%, 70%, 80%, 100%) for 30 seconds each and then in Citroclear for 5 minutes. Finally slides were mounted using DePex reagent (VWR International, UK). Controls for IGF-1R staining comprised sections of formalin-fixed, paraffin embedded cell pellets of SKUT-1 cells (IGF-1R deficient) and MCF-7 cells (overexpress IGF-1R).

2.2.3 TMA Scoring

Cores were scored according to the intensity and percentage of tumour tissue stained to create an immunoreactive score (IRS) for both membranous and cytoplasmic IGF-1R (Table 1). The final IRS for each tumour was calculated as the mean IRS of all cores scored. Total IGF-1R IRS was calculated as the sum of membranous and cytoplasmic scores for each tumour. TMAs were independently scored by OD and Dr Ketan Shah, Consultant Head and Neck Histopathologist. Discrepant scores were reviewed at conference microscope until a consensus was reached.

Score	Intensity	Percentage
0	No staining	0%
1	Weak staining	1-10%
2	Moderate staining	11-50%
3	Strong staining	51-80%
4		81-100%

Table 1: TMA scoring according to the intensity and percentage of tumour stained

The intensity and percentage of IGF-1R staining were scored according to the above scales. The product of the two scores gave the immunoreactive score (IRS) as described (Winter et al., 2006).

2.3 Cell Culture

All cell culture was performed in tissue culture hoods, using sterile plastic containers (Falcon). Cell were grown in Dulbecco's Modified Eagles Medium (DMEM), containing 10% Foetal Calf Serum (FCS) (Gibco), 1% Penicillin/Streptomycin (Gibco) and 1% Non-essential amino acids (Gibco). All cells were cultured in humidified cell culture incubators at 37 °C in 5% CO_2 and 95% air. Passage of cell lines was performed when cells reached 70-90% confluence. Cells were disaggregated using 0.25% Trypsin (Gibco), re-suspended in full medium, and seeded into new flasks. Early passage cells were frozen and banked for future use: Cells at 90% confluence were disaggregated as described above, and suspended in 1ml freezing medium (10% DMSO, 40% DMEM, 50% FCS). After culturing cells for 20-25 passages, cells were discarded and new early passage stock were recovered by rapidly thawing frozen cell suspensions, diluting in 50ml DMEM with 10% FCS and incubating overnight at 37 °C before changing the growth medium the following day.

2.4 Cell lines

The HNSCC cell lines SAS, CAL-27, BICR-56 and SCC-9 were obtained from Dr Stephan Feller (Weatherall Institute of Molecular Medicine, Oxford). The UM-SCC-50 and UM-SCC-99 cell lines were obtained under Material Transfer Agreement from Dr Thomas Carey (University of Michigan, United States). The UT-SCCO-60A cell line was obtained from Professor Reidar Grenman (University of Turku, Finland). Cell line characteristics are presented in Table 2. All cell lines were tested and found to be negative for mycoplasma (MycoAlert, Lonza Rockland Inc, Rockland, United States).

Cell line	Histological type	Tumour site	Primary tumour or metastasis
SAS	Squamous cell carcinoma	Anterior tongue	Primary
CAL-27	Squamous cell carcinoma	Anterior tongue	Primary
BICR-56	Squamous cell carcinoma	Anterior tongue	Primary
SCC-9	Squamous cell carcinoma	Anterior tongue	Primary
UM-SCC-50	Squamous cell carcinoma	Oropharynx	Primary
UM-SCC-99	Squamous cell carcinoma	Oropharynx	Primary
UT-SCC-60A	Squamous cell carcinoma	Oropharynx	Primary
Phoenix (T293)	Human Embryonic Kidney	N/A	Primary
MCF-7	Invasive breast ductal carcinoma	Breast	Secondary (pleural effusion)
SKUT-1	Leiomyosarcoma	Unknown	Primary

Table 2: Cell lines used for in-vitro work.

For cell line genotyping, genomic DNA was extracted from cells using a Wizard Genomic DNA Purification Kit (Promega, UK), according to the manufacturers instructions. The concentration of DNA was checked by spectrophotometry (Nanodrop, Thermo Scientific, UK), and 10 µl of genomic DNA at a concentration of 50ng/µl was submitted for genetic sequencing. Next generation sequencing was performed on genomic DNA from cancer cell lines to identify mutations in 46 genes, which are known to drive cancer pathways (Kanagal-Shamanna et al., 2014). Sequencing was performed by Dr Anthony Cutts in the Nuffield Department of

Clinical Laboratory Sciences, Oxford, using the IonTorrent Personal Genome Machine (LifeTechnology).

2.5 Treatments and reagents

2.5.1 Ligand treatment

Human recombinant IGF-1 ligand (Sigma-Aldrich, St Louis, USA) was used to activate IGF-1R. A stock solution of 110μM IGF-1 was made by diluting 1mg of IGF-1 powder in 1ml of 10mM hydrochloric acid (HCl). Single use aliquots were stored at -80°C. Activation of EGFR was achieved using human recombinant EGF ligand (Sigma-Aldrich). The EGF powder was diluted in 10mM acetic acid to a final concentration of 100 μg/ml. Single use EGF ligand aliquots of were stored at -80°C.

For ligand treatment, cells were washed twice with PBSA, and incubated overnight in serum free medium. The next day, cells were treated with 10nM ligand or HCl/acetic acid control in serum free DMEM for 15 minutes.

2.5.2 IGF-1R inhibitors

The IGF-1R tyrosine kinase inhibitor AZ12253801 was obtained from AstraZeneca. It was supplied in powder form and was reconstituted in DMSO to a stock concentration of 10mM. Aliquots were frozen at -20°C and were further diluted to a concentration of 1μM in DMEM with 10% FCS for cell treatments.

The IGF-1R tyrosine kinase inhibitor BMS-754807 was purchased from Bristol-Myers Squibb. It was provided in powder form and was made up to a stock concentration of 10mM in DMSO and stored in aliquots at -20°C. For cell treatments, it was further diluted in DMEM with 10% FCS to a concentration of 1μM.

2.5.3 EGFR inhibitor

The EGFR tyrosine kinase inhibitor Gefitinib was purchased from AstraZeneca as a powder, dissolved in DMSO to a concentration of 100mM and stored in aliquots at -20°C. For cell treatments it was diluted in DMEM with 10% FCS to a final concentration of 1μM.

2.5.4 Ionising radiation

Cells growing in 25cm^2 culture flasks were irradiated in a sealed source Caesium-137 irradiator (IBL 637 - CIS Bio International, France), in the Biomedical Sciences department at the John Radcliffe Hospital, Oxford. Cells were irradiated at doses of 2-10 Gy at a dose rate of 4.1 Gy/min.

Unless otherwise stated, three independent repeats of each experiment were performed, and pooled data were used to calculate SF_{50}, GI_{50} and dose-modifying factor (DMF) values.

2.6 Clonogenic Survival Assay

Clonogenic survival assays were performed in triplicate in 25cm^2 culture flasks. Optimum cell seeding density for each cell line was determined to achieve a final colony count of 800-1000 colonies per flask. Final seeding densities are shown in Table 3. For clonogenic survival assay, cells were seeded in DMEM + 10% FCS and incubated at 37°C overnight. The following day, culture medium was removed and cells were treated with drug or solvent control in fresh culture medium. Cells were then returned to the incubator. For irradiation assays, cells were irradiated 4 hours after drug treatment, and then returned to the incubator. After incubation for 5-10 days, to allow for formation of viable colonies of ≥ 50 cells in control flasks, cells were

fixed using 0.1% Coomassie Blue in 50% methanol and 7% acetic acid for 30 minutes. Flasks were washed with tap water, then distilled water and air-dried at room temperature. Automated colony counting was performed using Colcount (Oxford Optronix, UK), optimised to a manually counted control flask. Final colony counts were expressed as a percentage of solvent-treated controls, and were used to calculate the concentration of drug required to reduce the surviving fraction of colonies to 50% (SF50). Data from irradiation assays were used to calculate the dose-modifying factor (DMF):

$$\text{DMF} = \frac{\text{Irradiation dose required to achieve } SF_{50} \text{ without IGF-1R inhibitor (Gy)}}{\text{Irradiation dose required to achieve } SF_{50} \text{ with IGF-1R inhibitor (Gy)}}$$

Cell line	**Seeding density (cells per 25cm^2 flask)**	**Incubation duration**
SAS	1500	5 days
CAL-27	3000	6 days
BICR-56	5000	6 days
SCC-9	10,000	7 days
UM-SCC-50	10,000	10 days
UT-SCC-60A	10,000	7 days

Table 3: Seeding densities for HNSCC cell lines in clonogenic survival assay

2.7 Cell proliferation assay

Cells were seeded at densities of 500-1000 cells per well into u-clear 96-well plates. The following day, cells were treated with drug or solvent control and incubated at 37°C for 72 hours. Cell viability was determined using CellTiter-Glo Luminescent Assay (Promega). An equal volume of CellTiter-Glo reagent was added to media in wells, and plates were incubated in the dark on a rocker at room temperature for 30

minutes. Luminescence was measured using a Luminometer (Fluorostar Optima, BMG labtech). Values for treated cells were expressed as a percentage of those from solvent controls. Pooled data were used to calculate the concentration necessary to inhibit growth by 50% (GI_{50}).

2.8 Western Blotting

Culture medium was removed and cells were washed twice with cold PBSA. Adherent cells were scraped into cold PBSA and centrifuged at 3000rpm for 5 minutes. The supernatant was removed and the cell pellet was re-suspended in radio-immunoprecipitation assay (RIPA) lysis buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Triton, 150mM NaCl, 1mM EDTA and 20mM Tris pH7.5, supplemented with protease inhibitor cocktail, 1.5mM Pefabloc SC plus (Roche) and phosphatase inhibitor complex II and III (Sigma)). Samples were lysed on ice for 30 minutes, then centrifuged at 14,000 rpm for 10 minutes at 4°C to remove cell debris. Protein quantification against BSA standards was performed using the Pierce Bicinchonic Acid (BCA) assay (Thermo Scientific, UK). Absorbance at 562nm was measured using a µQuant spectrophotometer (Northstar Scientific, UK). For all samples, equivalent amounts of protein were denatured in 3X Laemmli buffer (70mM Tris pH 6.8, 5% βmercaptoethanol, 40% glycerol, 3% SDS and 0.05% bromophenol blue) at 100°C for 10 minutes. Between 50-80µg of protein was loaded onto 7.5%-14% polyacrylamide gels for SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein transfer to a nitrocellulose membrane (Hybond C extra, Amersham Biosciences) was achieved at 40mA per 8x10cm membrane for 3 hours, using a semi-dry transfer buffer (48mM Tris, 39mM glycine, 20% methanol, 1.3 mM SDS) in a transfer apparatus (Hoefer SemiPhor Transfer Unit, Amersham Biosciences).

Membranes were blocked in 5% non-fat dairy milk for 1 hour at room temperature, and primary antibody applied overnight. Primary antibodies are shown in Table 4. Following three 15 minute washes with Tris-buffered Saline and 0.1% Tween (TBS-T), bound antibody was detected using goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (DAKO, Ely, UK), and membranes were incubated at room temperature for 1 hour. Three further washes with TBS-T were performed and proteins were detected with Enhanced Chemiluminescence (ECLplus, Amersham Pharmacia Biotech), visualized on X-ray film or on a Gel Doc (BioRad, UK). Protein expression was quantified using ImageJ (Wayne Rasband, NIMH, Bethesda, US).

Antibody	Source	Primary Antibody dilution	Secondary antibody dilution	Blocking solution	Molecular weight (kDa)
Phospho-AKT Ser-473	Cell Signaling (#4051)	1:500	1:5000	BSA	60
Total AKT	Cell Signaling (#9272)	1:1000	1:10,000	BSA	60
β actin	Abcam (ab8224)	1:10,000	1:10,000	NFDM	42
DVL3	Cell Signaling (#3218)	1:1000	1:10,000	BSA	88
Phospho-ERK 1/2	Cell Signaling (#4377)	1:1000	1:10,000	BSA	42 & 44
Total ERK	Cell Signaling (#4695)	1:1000	1:10,000	BSA	42 & 44
Phospho-EGFR	Cell Signaling (#2236)	1:2000	1:10,000	BSA	180
EGFR	Cell Signaling (#2232)	1:2000	1:10,000	BSA	180
HRAS	Santa Cruz (sc-520)	1:500	1:5000	BSA	21
Phospho-IGF-1R β	Cell Signaling (#3024)	1:500	1:2000	BSA	95
IGF-1R β	Cell Signaling (#3027)	1:1000	1:10,000	NFDM	95
IGF2BP2	Thermo Scientific (PA5-29869)	1:1000	1:10,000	NFDM	66
Insulin Receptor β	Cell Signaling (#3025)	1:1000	1:10,000	NFDM	95
IRS-1	Cell Signaling (#2382)	1:1000	1:10,000	BSA	180
IRS-2	Upstate Cell Solutions	0.5μg/ml	1:10,000	BSA	180
Myosin IIb	Cell Signaling (#3404)	1:1000	1:10,000	BSA	230
Pan-RAS	EMD Millipore (OP40)	1:2000	1:10,000	BSA	21
$P16^{INK4A}$	BD Pharmingen (550834)	0.8μg/ml	1:5000	BSA	16
P53	Cell Signaling (#9282)	1:1000	1:10,000	BSA	53
PTEN	Cell Signaling (#9559)	1:1000	1:10,000	BSA	54

Table 4: Antibodies used for Western Blotting

NFDM = 5% Non-fat dairy milk, BSA = 5% Bovine serum albumin

2.9 Gene silencing with short interfering RNA (siRNA)

2.9.1 Forward transfection

The day before transfection, cells were seeded in 6 well plates at a density of 5 x 10^5 cells per well in 1.8ml DMEM+10% FCS. For transfection 5µl of DharmaFECT 1 in 95µl serum free DMEM was added to 5µl 20µM siRNA in 95µl serum free DMEM and complexes were incubated for 20 minutes at room temperature. Complexes were added in a dropwise fashion to the cells to give a final concentration of 50nM siRNA per well and incubated at 37°C for 48 hours.

2.9.2 Reverse transfection

For reverse transfection 1ml of cell suspension containing 5 x 10^5 cells in serum free DMEM was prepared in a 6 well plate. Complexes were prepared separately; 5µl siRNA and 5µl DharmaFECT 1 were added to 1ml of serum free DMEM, mixed gently then added to the cells to give a final concentration of 50nM siRNA per well. Cells were incubated at 37°C overnight, then the medium was replaced with DMEM + 10% FCS. The siRNAs used are shown in Table 5.

Name	Target	Source	Catalogue number
All Stars negative control	Human non-silencing control	Qiagen	1027281
HS_EGFR_10	Human EGFR	Qiagen	SI02660140
HS_EGFR_11	Human EGFR	Qiagen	SI02660147
IRS-2 siRNA	Human IRS-2	Santa Cruz Biotechnology	SC-29378

Table 5: Short interfering RNAs used for transfection of HNSCC cells

2.10 DNA transfection

2.10.1 Bacterial transformation

Wild type (WT) HRAS, HRAS G12V, HRAS G12D and empty vector (EV) plasmids were obtained in desiccated form from Professor Jeffrey N. Myers (MD Anderson Cancer Centre, Texas, US) (Hah et al., 2014). Plasmids were reconstituted in RNA-ase free water, competent JM109 e.coli were thawed on ice for 30 minutes, and 2 µl of each plasmid were added to competent cells, and incubated on ice for 30 minutes. Cells were heat-shocked at 42°C for 30 seconds and incubated on ice for 2 minutes. To each vial, 500 µl of Super Optimal Broth (SOB) medium was added, and cells were incubated on a shaker at 37°C for 1 hour. After incubation, 250µl of cell suspension was spread evenly over lysogeny broth (LB) agar plates containing 100µg ampicillin. Agar plates were incubated overnight at 37°C, then the following day a single colony from each plate was selected and inoculated into liquid culture.

Plasmid	Source
p-Babe empty vector (Addgene plasmid 1764)	JN Myers
p-Babe WT HRAS	JN Myers
p-Babe HRAS G12D	JN Myers
p-Babe HRAS G12V (Addgene plasmid 9051)	JN Myers

Table 6: HRAS plasmids used for DNA transfection

2.10.2 Plasmid DNA purification

Plasmid DNA was prepared using the EndoFree Plasmid MaxiPrep kit (Qiagen) according to the manufacturers instructions. In brief, an individual bacterial colony was selected and added to 10ml LB with 60µg/ml ampicillin and incubated on a shaker at 37°C overnight. The following day, a further 100ml of LB containing 60µg/ml ampicillin was added to cultures, which were then incubated at 37°C on a

shaker overnight. The bacterial cell suspension was centrifuged at 6000 x g for 15 minutes at 4°C, then the pellet was re-suspended in 10ml of buffer P1 and 1:1000 LyseBlue reagent. Then 10ml buffer P2 was added, lysates were mixed thoroughly and incubated at room temperature for 5 minutes, and 10ml chilled buffer P3 was added. Lysates were mixed well and transferred to QIAfilter cartridges and incubated at room temperature for 10 minutes before filtration. Buffer ER (2.5ml) was added to the filtered lysates, which were incubated on ice for 30 minutes and then applied to an equilibrated QIAGEN-tip 500. The QIAGEN-tip was washed twice with buffer QC, DNA was eluted with buffer QN and then precipitated with 10.5ml isopropanol. Tubes were centrifuged at 15,000 x g for 30 minutes at 4°C. The resulting supernatant was poured off and the DNA pellet washed with 5ml endotoxin-free 70% ethanol, and centrifuged at 15,000 x g for a further 10 minutes. Finally, the supernatant was removed, the DNA pellet was air dried and re-dissolved in 250µl endotoxin-free buffer TE. UV spectrophotometry (Nanodrop Technologies, Wilmington, US) was used to evaluate DNA concentration. Following purification, the wild-type HRAS, HRAS G12D and HRAS G12V plasmids were verified by Sangar sequencing (DNA sequencing service, Weatherall Institute of Molecular Medicine, Oxford), using the primers shown in Table 7.

	Sequence	**Source**
HRAS forward primer (5' to 3')	AGCAGGTGGTCATTGATGGG	Invitrogen
HRAS reverse primer (5' to 3')	TCACGCACCAACGTGTAGAA	Invitrogen

Table 7: Primers used for HRAS sequencing

2.10.3 Plasmid transfection

Phoenix cells are derived from transformed T293 Human Embryonic Kidney cells, which have been transfected with Moloney Murine Leukemia Virus (M-MLV) viral packaging proteins (Group Antigens, reverse transcriptase (gag-pol) and envelope proteins). These support the production of amphotropic retroviral vectors, which are used to infect other cell lines (Swift et al., 2001). Phoenix cells were grown in DMEM + 10% FCS and used when cells were at 70% confluence. Before transfection 7.5µl of filtered 20% glucose was added to 18µg of DNA in 22µl of distilled water. Then 9µl polyethylenimine (PEI, Sigma Aldrich) was added followed by 6ml DMEM + 10% FCS. Growth medium was removed from Phoenix cells growing in 75cm^2 flasks and replaced with the DNA mixture. Cells were incubated at 37°C overnight, then the medium was replaced with 7ml fresh growth medium. After 48 hours, the medium was harvested and centrifuged at 2000 x rpm to precipitate cell debris. The viral supernatant was divided into aliquots and stored at -80°C.

2.10.4 Infection of HNSCC cell lines with viral DNA

For infection of HNSCC cells with viral DNA, neat viral medium supplemented with 8µg/ml Polybrene (Millipore, Billerica, US) was applied to cells overnight. The following day, the medium was changed for fresh growth medium, which was then supplemented with 1.5µg/ml Puromycin (Sigma-Aldrich, St. Louis, US) 72 hours after initial infection. Puromycin-resistant pools of cells were expanded in culture. To boost transgene expression, puromycin resistant cultures were re-infected with HRAS constructs, three days prior to harvesting cells.

2.11 Statistical analysis

The statistical analysis of survival and functional outcomes in surgically treated OPSCC has been previously described (Sections 2.1.2 & 2.1.3). Scores from IGF-1R immunostaining were entered on to a spreadsheet (Microsoft Excel, Microsoft, WA, USA). Differences in IGF-1R expression between matched normal and tumour tissue were determined using a paired t-test (two-tailed). The association between IGF-1R IRS and tumour T stage or HPV status were tested using a Mann-Whitney U Test (Prism v6.0, Graphpad, USA). Kaplan Meier survival analysis, univariate and multivariate analyses were performed using Prism (v6.0, Graphpad, USA) and Stata (version 11, Stata Corporation, TX, USA). Data derived from cell line work were entered onto spreadsheets (Microsoft, WA, USA), and analysed using Prism (v6.0, Graphpad, US). Data points on graphs represent mean values, error bars represent standard error of the mean (SEM). The student's T test was used to compare two groups of data. Statistical analyses were performed by Dr Cheng Han (Department of Oncology, Oxford University).

3 Survival and functional outcomes in surgically treated oropharyngeal cancer

3.1 Introduction

Although the incidence of HNSCC has fallen slightly over the last three decades, the incidence of oropharyngeal squamous cell carcinoma has risen sharply (Howlader N, 2013, Mehanna et al., 2010a). This is thought to relate to an increase in the prevalence of HPV related disease (Chaturvedi et al., 2011). HPV positive OPSCC represents a biologically distinct subtype of HNSCC with improved survival outcomes compared to HPV negative disease (Adelstein and Rodriguez, 2010, Rieckmann et al., 2013, Ang et al., 2010). As the relative proportion of HPV positive OPSCC has increased, treatment paradigms have shifted away from surgery towards primary chemoradiotherapy, reflecting the increased radiosensitivity of the disease (Chen et al., 2013). Despite this however, surgery remains and accepted treatment modality. A large national trial (PATHOS trial) is currently underway, investigating treatment modality and survival outcome in OPSCC, but this is unlikely to report results for several years. The majority of data currently available on survival outcomes in OPSCC are therefore derived from small retrospective series, which have limited follow up duration and do not report on HPV status. In addition, few studies report on long-term functional outcomes in the HPV era. The first part of this project aims to define the functional and survival outcomes from surgically treated patients with oropharyngeal cancer.

3.2 Survival outcomes in surgically treated OPSCC

3.2.1 Patient demographics

Two hundred and four patients with OPSCC, treated with primary surgery between 1st January 2000 and 31st December 2009 were identified from the Oxford Head and Neck cancer database. Thirty-six of these patients did not meet the inclusion criteria for the study and were excluded: Thirteen patients were lost to follow up after primary surgery, seven were not treated with primary surgery and in six cases the sub-site was not the oropharynx. In five cases patient case notes were lost, three cases had non-SCC pathology and two were treated with palliative intent. Therefore, 168 patients were eligible and were included in the study. Not all of these cases were suitable for inclusion in the TMA: In 26 cases, there was insufficient primary tumour for TMA construction, in 19 cases tissue blocks were not compatible with the automated TMA construction process and in seven cases no primary tumour specimens were available. In five cases, no SCC was evident in the resection specimens and in four cases histopathology slides and blocks were missing. Therefore, 107 cases were suitable for inclusion in the TMA, HPV status determination and survival analysis (Figure 2).

Patient demographic data are presented in Table 8. The majority of patients were male (n=77, 72%), and most presented with locally advanced disease (n=97, 91% UICC stage III or IV). The commonest sub-site affected within the oropharynx was the tonsil (n=69, 64%) followed by the tongue base (n=28, 26%). All patients underwent surgery as the primary treatment modality. The predominant surgical approach was a midline lip splitting mandibulotomy (82%, n=88) with free flap reconstruction (n=81, 76%). One hundred patients received adjuvant radiotherapy (93%), with a median dose of 60Gy in 30 fractions (range 56Gy-66Gy, 28-33

fractions). Seventeen patients (16%) received platinum based chemotherapy in combination with radiotherapy as adjuvant treatment, with patients receiving a median of two cycles (range 1-3 cycles, no chemotherapy data available in 13 cases).

Following P16 immunohistochemistry and in-situ hybridisation for HPV DNA, 40% of patients (n=41) were characterised as HPV positive (Table 9). Histopathological examination of tumour specimens demonstrated extracapsular spread in 54 cases (50%), perineural spread in 23 cases (22%) and lymphovascular invasion in 25 cases (23%) (Table 8).

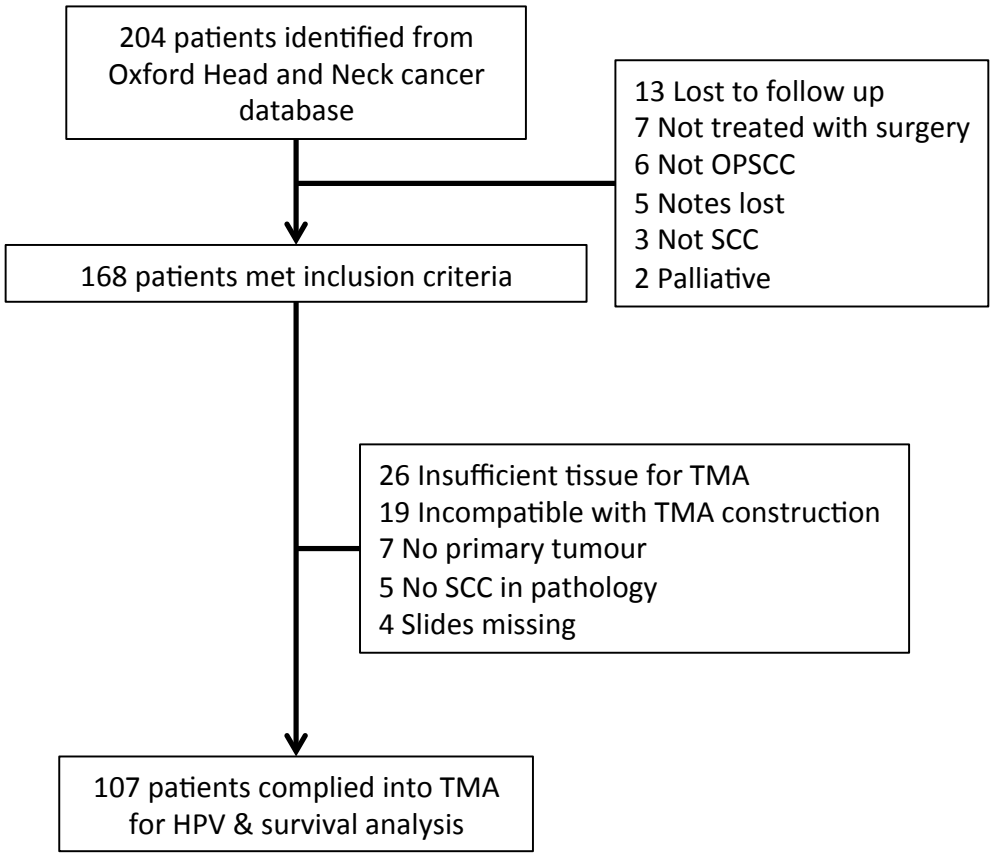


Figure 2: Flowchart showing patient selection for survival analysis.

Two hundred and four patients were identified from the Oxford Head and Neck Cancer Database, of which 36 did not meet the inclusion criteria for the study and were excluded. In 61 cases, tissue was not suitable for inclusion in the tissue microarray (TMA) therefore 107 cases were compiled to form the TMA.

	Number of patients (%)
Total No.	107
Gender:	
Male	77 (72%)
Female	30 (28%)
T stage:	
1	15 (14%)
2	50 (47%)
3	19 (18%)
4	23 (21%)
N stage:	
0	25 (23%)
1	16 (15%)
2a	14 (13%)
2b	43 (40%)
2c	4 (4%)
3	5 (5%)
AJCC Stage:	
1	1 (1%)
2	9 (8%)
3	18 (17%)
4	79 (74%)
Tumour site:	
Tonsil	69 (64%)
Base of tongue	28 (26%)
Posterior pharyngeal wall	1 (1%)
Soft palate	3 (3%)
Overlapping lesion of oropharynx	6 (6%)
Resection type:	
Transoral resection (non-laser)	8 (7%)
Transoral laser resection	11 (10%)
Lip split mandibulotomy	88 (82%)
Reconstruction type:	
No reconstruction	23 (21%)
Local flap	1 (1%)
Pectoralis major flap	2 (2%)
Free flap:	81 (76%)
Radial forearm	44 (41%)
Anterolateral thigh	11 (10%)
Ulnar forearm	13 (12%)
Vertical Rectus Abdominus (VRAM)	5 (45%)
Not specified	8 (7%)
Radiotherapy Type:	
None	7 (7%)
Adjuvant	100 (93%)
Chemotherapy Type:	
None	90 (84%)
Adjuvant chemotherapy	17 (16%)

Table 8: Demographic details for patients with oropharyngeal cancer treated with primary surgery in Oxford between January 2000 and December 1999.

		HPV DNA ISH		
		Negative	**Positive**	**Total**
P16 IHC	**Negative**	30 (29%)	0 (0%)	30 (29%)
	Positive	31 (30%)	41 (40%)	72 (71%)
Total		61 (60%)	41 (40%)	102 (100%)

Table 9: HPV status determination.

Results from P16 immunohistochemistry (P16 IHC) and in-situ hybridisation for HPV DNA (HPV DNA ISH) 102 patients with OPSCC treated with primary surgery. Patients were considered to be HPV positive if both P16 IHC and HPV DNA ISH were positive. Figures represent number of cases (percentage). In 5 cases HPV data were not available.

	Yes	**No**	**Data not available**
Extracapsular Spread (ECS)	54 (50%)	36 (34%)	17 (16%)
Perineural spread (PNS)	23 (21%)	69 (64%)	15 (14%)
Lymphovascular invasion (LVI)	25 (23%)	67 (63%)	15 (14%)

Table 10: Histopathological outcomes

Histopathological data from routine reporting of pathology specimens of 107 patients with oropharyngeal cancer treated with primary surgery in Oxford.

3.2.2 Survival outcomes

Seventy patients (65%) were alive at final follow-up. Kaplan-Meier analysis was performed to determine 5-year and 10-year estimates of overall and disease specific survival. At a mean follow-up duration of 59 months (range 0 to 168 months) 5-year and 10-year estimates for overall survival were 68% and 66% respectively. Estimates for 5-year and 10-year disease specific survival were 78% and 75% (Figure 3A). Stratification of patients according to both HPV ISH and P16 status showed significantly higher 5-year overall survival in patients with P16 positive and HPV ISH positive disease (5-year OS: P16 positive 76%, P16 negative 49% (p=0.003), HPV ISH positive 85%, HPV ISH negative 57% (p=0.002)) (Figure 3B & Figure 3C). Disease specific survival was also higher in both P16 and HPV ISH positive patients at 5 years (5-year DSS: P16 positive 84%, P16 negative 65% (p=0.027), HPV ISH positive 91%, HPV ISH negative 69% (p=0.012)).

Log-rank testing was used to identify significant associations between clinical variables and survival outcomes. This showed that higher tumour T stage, being a current smoker, and the presence of perineural spread and lymphovascular invasion were associated with reduced disease specific and overall survival (Table 11). The log rank test is purely a measure of statistical significance and therefore the effect size was not calculated for these associations.

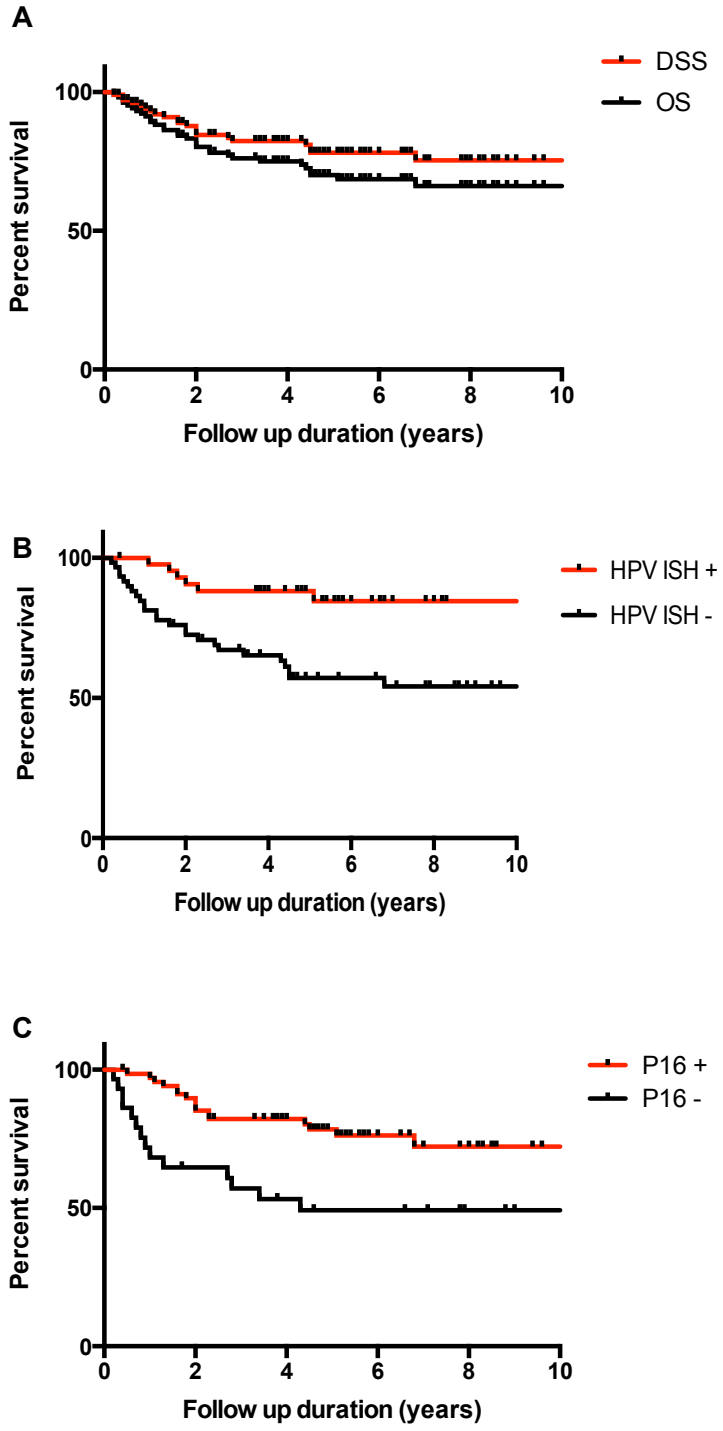


Figure 3: Kaplan-Meier estimates of overall survival in patients with OPSCC treated with primary surgery (n=107)

A) Overall survival (OS) and disease specific survival (DSS) in all patients. 5-year OS 68%, DSS 78%. 10-year OS 66%, DSS 75%.
B) 5-year OS is significantly higher in patients with HPV ISH positive (HPV ISH+) tumours (5-year OS: HPV ISH+ 85%, HPV ISH- 57%, p=0.002)
C) 5-year OS is significantly higher in patients with P16 positive (P16+) tumours (5-year OS: P16+ 76%, P16- 49%, p=0.003)

	Overall survival (p-value)	Disease specific survival (p-value)
HPV status (HPV+ vs HPV-)	0.002	0.014
Tumour T stage (T1/2 vs T3/4)	< 0.001	0.030
Tumour N stage (N0 vs N1-3)	0.094	0.102
AJCC Stage group (Stage I/II vs stage III/IV)	0.045	0.926
Smoking (current vs non smoker)	< 0.001	0.021
Extracapsular spread (present vs absent)	0.870	0.803
Lymphovascular invasion (present vs absent)	0.003	0.001
Perineural spread (present vs absent)	0.007	0.002

Table 11: Associations between clinico-pathological variables and survival

Associations between clinico-pathological variables and survival were calculated using a log-rank test. Where data were missing, patients were excluded from the analysis.

3.2.3 Discussion

In the UK in 2012, 2303 cases of OPSCC underwent treatment with curative intent, of which 37% were treated with primary surgery (HQIP National Head and Neck Cancer Audit - Eight annual report, 2012). But despite the widespread use of primary surgery in the treatment of OPSCC, little data exist on long-term survival in this group of patients in the HPV era. This study presents survival data for 107 patients with OPSCC and a mean follow-up duration of 59 months. The 5-year overall survival and disease specific survival estimates in this series are consistent with previously published data, although there is wide variation in survival outcomes previously reported (Parsons et al., 2002, Rich et al., 2009).

There may be several reasons for the wide variation in reported survival outcomes in OPSCC. Firstly, some studies report outcome data for a subset of patients with oropharyngeal cancer, such as patients with advanced disease (Al-Mamgani et al., 2013, Calais et al., 1999, Nguyen et al., 2007), or with tumours affecting one particular oropharyngeal sub-site (Grant et al., 2006, Zhen et al., 2004). Data from this and other studies indicate that advanced disease and oropharyngeal sub-site affect survival outcome, and care should therefore be taken before drawing direct comparison between studies.

Another explanation for the wide range of survival outcomes reported in OPSCC, is the variation in both the reporting of HPV data and the proportion of HPV related disease. The prognostic influence of HPV in HNSCC was identified in 1992, but routine testing for HPV infection in HNSCC did not become commonplace until much later (Snijders et al., 1992). As a result, several retrospective studies reporting outcomes in OPSCC lack HPV data and are thus difficult to interpret (Chen et al., 2013, Kano et al., 2013, Parsons et al., 2002, Zhen et al., 2004). In addition,

evidence suggests that the incidence of HPV associated OPSCC has increased significantly over the last 20-30 years, meaning that comparison between recent and historic survival data may not be valid (Schache et al., 2011, Mehanna et al., 2010a). For those studies that do report HPV status, the HPV detection method may provide another confounding variable. P16 immunohistochemistry, reverse Polymerase Chain Reaction and in-situ hybridisation for HPV DNA have all been used for the detection of HPV in OPSCC, but when used alone, none are 100% sensitive or specific, and all may yield different results (Evans et al., 2013, Haughey et al., 2011, Rich et al., 2009).

In the UK, the majority of patients with OPSCC are treated with primary chemoradiotherapy, and clinical trials are currently underway which aim to compare survival outcomes in different treatment modalities. Existing survival data are derived from un-matched retrospective analyses and should therefore be interpreted with caution. O'Connell et al conducted a retrospective analysis of 344 patients in the United States with advanced OPSCC and stratified survival outcomes according to primary treatment modality (O'Connell et al., 2013). They found that patients receiving surgery with adjuvant chemoradiotherapy had significantly better overall survival at 5 years than those receiving surgery and adjuvant radiotherapy or chemoradiotherapy alone. The proportion of patients with HPV positive tumours in each treatment group are however, not reported. Evans et al conducted a similar retrospective analysis of survival outcomes for 126 patients treated for OPSCC in the UK. The authors found that crude overall survival was higher in surgically treated patients than those receiving primary chemoradiotherapy, but the surgery group contained more patients that were HPV positive. When Cox regression analysis was employed to control for HPV status, there was no significant difference in overall survival at 5 years between the two groups. This again highlights the importance of including HPV data when reporting survival outcomes in HNSCC.

The results from this study confirm the association between survival and known prognostic indicators in HNSCC including HPV status, tumour T stage, smoking status, lymphovascular invasion and perineural spread of tumour. Some other established prognostic factors, however, were not associated with survival in this series. For example, neither nodal stage, nor the presence of extracapsular spread in lymph node metastases were prognostic. These results appear to contradict the findings of previous studies, which indicate that both N+ disease and the presence of ECS are associated with significantly reduced survival (Layland et al., 2005, Dunne et al., 2006). An explanation for this may lie in the high proportion of HPV positive cases in this cohort (42%). Recent work by Haughey demonstrates that clinical factors associated with survival outcome in HPV negative disease are non-prognosticators in HPV positive disease. In particular, nodal status and the presence of ECS are not associated with survival outcome in this patient group (Haughey and Sinha, 2012).

The results from this survival analysis have demonstrated the significant mortality associated with OPSCC, in particular in HPV negative disease. The relative chemoradioresistance and adverse survival outcomes in HPV negative OPSCC make the investigation of novel treatment approaches in this group a high priority. Work presented in Chapters 4 & 5 will aim to address this issue.

3.3 Functional outcomes in surgically treated OPSCC

3.3.1 Patient demographics

Evaluation of functional outcomes was performed in surviving patients, treated for biopsy proven OPSCC with primary surgery ± radiotherapy ± chemotherapy in Oxford between 1st January 2000 and 31st December 2009. Two-hundred and four patients were identified from the Oxford Head and Neck Cancer Database, and 36 were excluded as previously described (section 3.2.1). Of the 168 patients suitable for inclusion in the study, 61 (36%) died over the course of the study period, leaving 107 surviving patients (64%). The MD Anderson Dysphagia Inventory (MDADI) and the University of Washington Quality of Life (UWQoL) questionnaire were sent to all 107 surviving patients and responses were received from 72 patients (65%). The mean time from completion of treatment was 93 months (range 53-165 months).

Demographic and treatment details for patients included in the functional outcome analysis were as follows (Table 12); Fifty-six patients (78%) were male and 16 were female (22%). The majority presented with advanced disease (UICC stage III or IV, n=65, 90%). The OPSCC primary site was tonsil in 49 cases (68%) and tongue base in 19 cases (26%). Approximately one-third of patients (n=26, 36%) underwent transoral surgery, and two-thirds (n=46, 64%) received a lip-splitting mandibulotomy and for tumour resection. Most patients (n=69, 96%) received adjuvant radiotherapy with a median dose of 60Gy in 30 fractions (range 60Gy to 66Gy in 30 to 33 fractions), and 16 patients (22%) received adjuvant chemotherapy concurrent with radiotherapy. During this study period no patient received intensity modulated radiotherapy (IMRT).

	Number of patients (%)
Total No.	72
Gender:	
Male	56 (78%)
Female	16 (22%)
T stage:	
1	23 (32%)
2	35 (49%)
3	5 (7%)
4	9 (13%)
N stage:	
0	12 (17%)
1	9 (13%)
2a	13 (18%)
2b	31 (43%)
2c	4 (6%)
3	3 (4%)
Stage group:	
1	3 (4%)
2	4 (6%)
3	7 (10%)
4	58 (81%)
Tumour site:	
Tonsillar fossa	1 (1%)
Tonsil	49 (68%)
Base of tongue	19 (26%)
Posterior pharyngeal wall	1 (1%)
Soft palate	1 (1%)
Overlapping lesion of oropharynx	1 (1%)
Resection type:	
Laser resection	26 (36%)
Lip splitting mandibulotomy	46 (64%)
Reconstruction type:	
No reconstruction	27 (38%)
Free flap	45 (63%)
Radiotherapy Type:	
None	3 (4%)
Adjuvant	69 (96%)
Chemotherapy Type:	
None	56 (78%)
Adjuvant chemoradiotherapy	16 (22%)
Gastrostomy tube status:	
No	56 (78%)
Yes	16 (22%)

Table 12: Demographic and treatment details for patients included in functional outcome analysis.

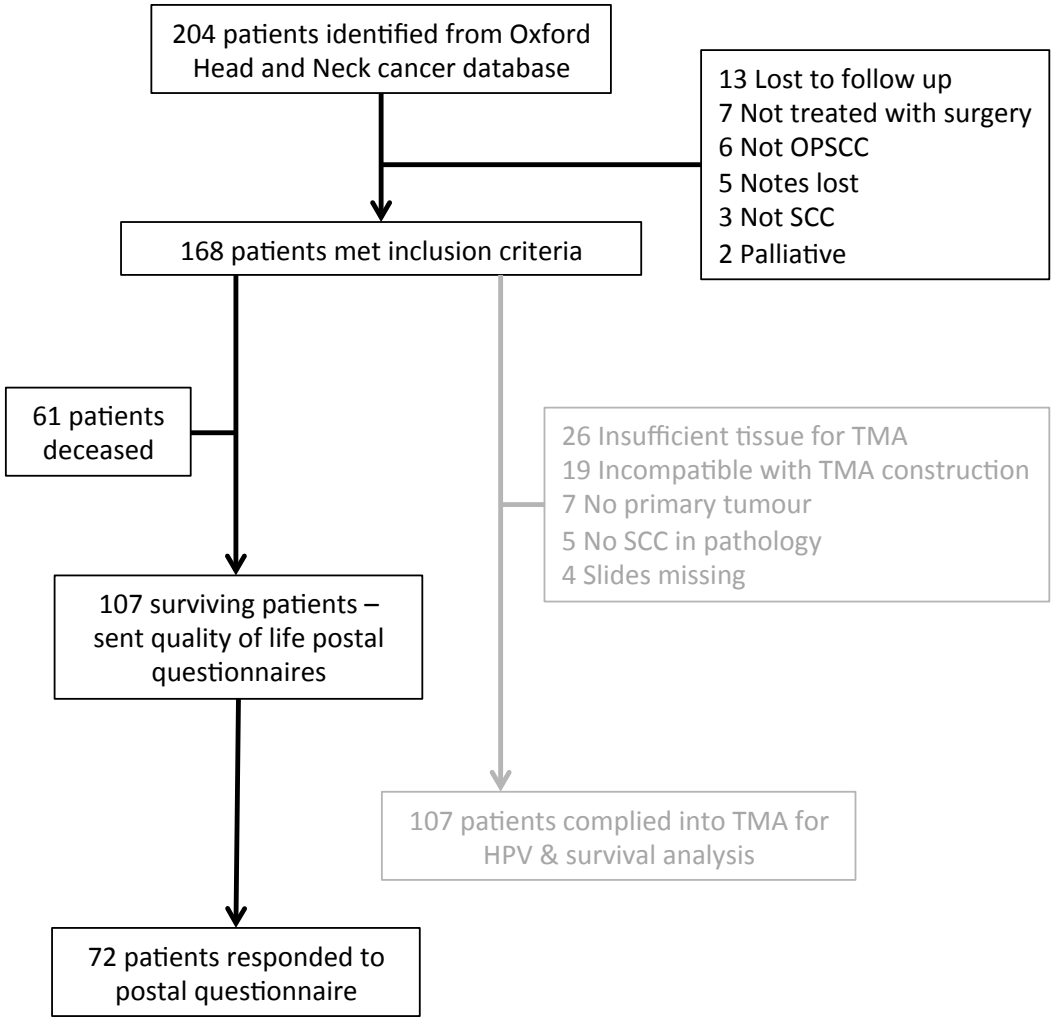


Figure 4: Flowchart showing patient selection for functional outcome analysis.

Two hundred and four patients were identified from the Oxford Head and Neck Cancer Database, of which 36 did not meet the inclusion criteria for the study and were excluded. Of the 168 patients included in the study, 61 died during the follow-up period and were not included in the functional outcome analysis. Postal questionnaires were therefore sent to 107 patients, of which 72 responded.

3.3.2 Functional outcomes

The UWQoL and MDADI questionnaires are disease specific, validated tools for determining patient reported outcomes in HNSCC. The UWQoL is made up of 12 questions, categorised by Rogers et al as either physical or social/emotional. For analysis and outcome reporting, it is recommended that mean composite physical and social/emotional scores are used (Rogers et al., 2010). Similarly, Chen et al suggest grouping individual questions in the MDADI into physical, functional, emotional and global subscales, and reporting mean outcomes for each of these domains. Other authors support the use of a single mean total MDADI score (Guedes et al., 2013). In this study, mean composite physical and social/emotional scores from the UWQoL questionnaire and mean domain and total scores from the MDADI are used as outcome measures.

Mean scores for each question of the UWQoL questionnaire, and composite scores for physical and social/emotional function are shown in Table 13. Scores for each domain of the MDADI and the total mean score are shown in Table 14. Sixteen patients in this series (22%) underwent gastrostomy tube insertion. At one year following treatment one patient had a gastrostomy tube in-situ (Table 15). The mean duration of gastrostomy tube feeding was 114 days (range: 7-484 days).

In order to examine the impact of treatment modality on quality of life and functional outcomes, patients were stratified according treatment and scores from the UWQoL and MDADI presented (Table 16). The outcomes of the UWQoL and MDADI questionnaires varied according to primary and adjuvant treatment modality. This initial analysis suggests that clinical factors including treatment modality may influence functional and quality of life outcomes in surgically treated OPSCC.

UWQoL question	Mean score (Standard Deviation)
Taste	75.3 (28.7)
Saliva	53.6 (30.4)
Appearance	71.9 (15.7)
Chewing	73.9 (28.9)
Speech	82.5 (18.4)
Swallowing	75.2 (18.1)
Composite Physical Score	**72.2 (15.1)**
Activity	78.5 (19.5)
Shoulder	72.8 (26.6)
Recreation	85.2 (16.7)
Pain	81.3 (23.1)
Mood	79.9 (22.8)
Anxiety	81.0 (22.0)
Composite Social/Emotional Score	**80.0 (15.9)**
QoL: current vs. pre-morbid	49.3 (23.7)
QoL last 7 days	66.2 (20.7)
Overall QoL	68.5 (20.5)

Table 13: University of Washington Quality of Life (UWQoL) questionnaire scores

Mean scores for each question of the UWQoL questionnaire from 72 patients treated for OPSCC with primary surgery. (QoL = quality of life).

MDADI domain	Mean score (Standard Deviation)
Global	72.1 (25.6)
Emotional	73.5 (19.7)
Functional	81.1 (19.4)
Physical	68.4 (19.9)
Mean total MDADI score	73.9 (18.8)

Table 14: MD Anderson Dysphagia Inventory (MDADI) scores

Mean scores from each domain of the MDADI in 72 patients with OPSCC treated with primary surgery.

	Gastrostomy feeding tube inserted	Gastrostomy feeding tube present at 1 year
Yes	16 (22%)	1 (1%)
No	56 (78%)	71 (99%)

Table 15: Number of patients undergoing gastrostomy tube insertion

Gastrostomy tube insertion data were collected from the department of Dietetics database. Gastrostomy tubes were placed according to the local departmental protocol in patients receiving bilateral irradiation of the neck or oropharynx.

	UWQoL		MDADI				
Treatment modality	**Physical**	**Social Emotional**	**Global**	**Emotional**	**Functional**	**Physical**	**Mean MDADI**
Mandibulotomy + RT (n=38)	66.8 (15.7)	77.5 (17.7)	66.0 (27.0)	67.0 (20.9)	74.8 (21.2)	61.4 (19.1)	67.3 (19.8)
Mandibulotomy + CRT (n=6)	75.5 (9.4)	86.1 (8.3)	80 (21.9)	75.6 (10.9)	90 (11.8)	62.8 (12.4)	77 (9.8)
Transoral resection + RT (n=15)	75.8 (11.2)	75.6 (14.5)	73.3 (24.7)	77.5 (17.9)	87.7 (14.5)	78.1 (18.1)	79.1 (16.4)
Transoral resection + CRT (n=10)	84.4 (12.4)	89.6 (8.7)	88 (16.9)	90 (14.3)	90 (16.3)	85.4 (16.2)	88.1 (14.3)

Table 16: Results from the UWQoL and MDADI questionnaires stratified by treatment modality

Patients were stratified according to primary and adjuvant treatment modality, figures represent mean values (SD). Patients receiving surgery alone (either transoral surgery or mandibulotomy) are not presented due to the low number of patients in this group (n=3, 4%).

In order to determine whether clinical factors were associated with functional outcome in OPSCC, bivariate analysis was performed (Table 17). This suggested that specific clinical and treatment variables were associated with functional outcome in patients with surgically treated OPSCC. In particular, increasing age, larger tumour size, open surgery and free flap reconstruction were all significantly associated with lower scores in the MDADI and UWQoL questionnaires. By contrast, treatment with adjuvant chemotherapy was associated with higher scores in both questionnaires. To identify which of these factors were independently associated with outcome in OPSCC, a multivariate regression analysis was performed (Table 18). This showed that increasing age was independently associated with reduced composite scores in the physical ($p=0.001$), social/emotional ($p=0.002$) and overall QoL ($p=0.01$) domains of the UWQoL questionnaire, as well as reduced functional ($p=0.015$), physical ($p=0.022$) and mean ($p=0.014$) MDADI scores. Larger tumours were significantly associated with poorer global ($p=0.037$), emotional ($p=0.004$) and functional ($p=0.012$) MDADI outcomes. The type of surgery performed also had a significant effect on functional outcome. Open surgery was associated with reduced physical domain ($p<0.001$) and mean total ($p=0.002$) MDADI scores, and free flap reconstruction was associated with a reduced physical composite UWQoL score ($p=0.006$). Conversely, patients receiving adjuvant chemotherapy had significantly higher MDADI global ($p=0.047$) and emotional ($p=0.025$) scores.

	Age	Stage Stage 1/2 vs stage 3/4	Resection Transoral vs open surgery	Reconstruction No reconstruction vs free flap	Chemotherapy None vs adjuvant chemotherapy
UWQoL					
Physical function	<0.001 (-)	0.033 (-)	0.008 (-)	0.006 (-)	0.009 (+)
Social/emotional function	0.002 (-)				0.037 (+)
Overall QoL	0.006 (-)	0.043 (-)			
MDADI					
MDADI Global		0.018 (-)			0.023 (+)
MDADI Emotional		0.001 (-)	0.002 (-)	0.003 (-)	0.010 (+)
MDADI Functional	0.007 (-)	0.013 (-)	0.021 (-)	0.024 (-)	0.015 (+)
MDADI Physical	0.048 (-)	0.015 (-)	< 0.001 (-)	< 0.001 (-)	
Mean MDADI score	0.023 (-)	0.008 (-)	0.003 (-)	0.003 (-)	0.014 (+)

Table 17: Bivariate analysis of functional outcomes and clinical factors in patients with surgically treated OPSCC

Clinical variables and functional outcome data from the MDADI and UWQoL questionnaires were entered into contingency tables and analysed using Pearson's chi-square test or Fisher's Exact test. Only significant associations are shown. Numbers represent p values, and the nature of the association is indicated in parentheses: (+) positive association, (-) inverse association. (QoL = quality of life).

	Age	**T Stage** (T1/2 vs T3/4)	**Resection** (Transoral vs open surgery)	**Reconstruction** (None. vs free flap)	**Chemotherapy** None vs adjuvant chemotherapy
UWQoL					
Physical Function	-0.59 (-0.92 to -0.25) p=0.001			-9.29 (-15.77 to -2.80) p=0.006	
Social/ Emotional Function	-0.61 (-0.98 to 0.24) p=0.002				
Overall QoL	-0.58 (-0.95 to 0.05) p=0.010				
MDADI					
Global		-16.02 (-31.07 to -0.98) p=0.037			14.14 (0.21 to 28.07) p=0.047
Emotional		-16.66 (-27.75 to -5.57) p=0.004			11.76 (1.49 to 22.03) p=0.025
Functional	-0.56 (-1.01 to 0.11) p=0.015	-14.24 (-25.24 to -3.23) p=0.012			
Physical	-0.50 (-0.93 to 0.08) p=0.022		-19.08 (-27.53 to -10.63) p<0.001		
Mean MDADI	-0.54 (-0.97 to 0.11) p=0.014		-13.50 (-21.91 to -5.09) p=0.002		

Table 18: Multivariate regression analysis of clinical variables and functional outcomes in patients with surgically treated OPSCC.

Significant associations identified in the bivariate analysis of clinical variables and functional outcomes were entered into multivariate regression analysis. Only significant associations are shown. Figures indicate the beta coefficient, (95% confidence interval) and p value. (QoL = quality of life)

3.3.3 Discussion

This study reports long-term functional outcome data for a group of patients with OPSCC treated with primary surgery. Of 107 patients alive at final follow up, 72 (65%) responded to the postal questionnaires. This response rate is similar to that seen by Thomas and colleagues in a comparable cohort (66%), and highlights the challenges associated with data collection after an extended follow up period (mean time from completion of treatment = 93 months, range 53-165 months) (Thomas et al., 2008).

The mean scores from both the MDADI and UWQoL questionnaires shown in Table 13 & Table 14 indicate a moderate long-term reduction in quality of life and functional performance status following primary surgery for OPSCC. These findings are consistent with previous data, which demonstrate the morbidity associated with the surgical treatment of OPSCC (McConnel et al., 1998, Zafereo et al., 2010). Despite reduced scores in quality of life questionnaires, however, only one patient in this series was gastrostomy tube dependent at one year following completion of treatment, and none had a gastrostomy in-situ at final follow up (Table 15). Gastrostomy tube feeding has previously been used as a surrogate marker of swallowing function following treatment for HNSCC (Skoner et al., 2003, Zafereo et al., 2010). The absence of long-term gastrostomy tube dependent patients in this series is therefore consistent with the mean 'swallowing' subscale score of the UWQoL, which indicates moderate to good levels of swallowing function in this group of patients (Table 13) (Thomas et al., 2008).

Direct comparison of functional outcomes from this study with those of other studies is difficult due to differences in outcome measures used, patient characteristics and follow up duration. All of these factors may influence patient reported outcome

measures, and may contribute to the wide variation in quality of life outcomes observed in other studies.

In a series of 65 patients with OPSCC treated with surgery, Zafereo and colleagues report that 65% of patients were gastrostomy tube dependent at a mean follow up duration of 36 months. In that study however, a higher proportion of patients had large tumours and tumours affecting the tongue base than in the current study, both of which are associated with adverse functional outcome (Dwivedi et al., 2012). By contrast, Tulunay-Ugur and colleagues published gastrostomy feeding data in a group of 243 patients with HNSCC treated with primary chemoradiotherapy. Although some of these patients had tumours affecting other sub-sites of the head and neck, 62% were treated for OPSCC. They found that 37% of patients required gastrostomy tube feeding for longer than 12 months (Tulunay-Ugur et al., 2013). The rates of feeding tube dependency in this study are therefore lower than in some other studies, but as previously mentioned this may relate to differences in study design and local policies for gastrostomy tube insertion and feeding.

The UWQoL questionnaire has previously been used to evaluate outcomes in OPSCC. Dwivedi et al performed a cross-sectional study of 38 patients with OPSCC treated by primary surgery ± adjuvant chemoradiotherapy. The results showed mean UWQoL swallowing sub-scale and total composite scores of 76 and 73 respectively. These are similar to the mean UWQoL outcomes in this study (swallowing sub-scale score = 75, total composite score = 76) (Dwivedi et al., 2012).

The MDADI has also been used in this context. Gillespie et al. performed a cross-sectional study of 21 patients with OPSCC undergoing either surgery or chemoradiotherapy. In the group treated with primary surgery MDADI domain scores were as follows: global 56.4, emotional 59.4, functional 62.5 and physical 52.5. In patients treated by chemoradiotherapy, MDADI scores were higher in all domains,

although the number of patients in each arm of this study is small (n=10, n=11) (Gillespie et al., 2004).

Bivariate analysis of clinical variables and questionnaire outcomes identified several factors, which were associated with quality of life in patients with OPSCC. These associations, however, may be influenced by confounding relationships between key variables. For example, larger tumours may require an open surgical approach and free flap reconstruction, while smaller tumours may be resected via a trans-oral approach and may not require reconstruction. Multivariate analysis therefore aimed to identify clinical variables that are independently associated with quality of life outcomes in OPSCC.

Following multivariate analysis, increasing age was significantly associated with reduced quality of life in several domains of both the UWQoL and MDADI questionnaires. These data are consistent with previous reports in OPSCC indicating that increasing age at the time of diagnosis is associated with reduced functional outcome following treatment (Skoner et al., 2003). Increased tumour T stage was another factor associated with reduced quality of life outcome scores in multivariate analysis. Higher tumour T stage is indicative of increasing tumour size, and larger resection volume during surgery for OPSCC. This has previously been shown to be an important predictor of swallowing function after treatment for tumours of the tongue base (Pauloski et al., 2004).

In addition to increasing tumour T stage, open surgery and free flap reconstruction were associated with reduced quality of life outcome scores in multivariate analysis. Few studies have compared quality of life outcomes in trans-oral and open surgery for OPSCC. There is, however evidence to suggest that trans-oral surgery may lead to better swallowing outcomes than open surgery in the immediate post-operative period (Williams et al., 2013). Similar data indicate that free flap reconstruction of

oropharyngeal defects following tumour resection is associated with adverse swallowing outcomes (McConnel et al., 1998).

In this study, patients receiving adjuvant chemotherapy in addition to radiotherapy had significantly higher global and emotional MDADI scores than patients receiving adjuvant radiotherapy alone, indicating higher quality of life outcomes. These results seem to contradict the findings of some previous studies, which suggest that the addition of chemotherapy to adjuvant radiotherapy causes higher levels of toxicity in patients with HNSCC (Adelstein et al., 2003, Cooper et al., 2004). In a Phase III trial in 295 patients with HNSCC, Adelstein and colleagues showed that patients receiving concomitant CRT had a significantly higher rate of early grade 3 toxicity reactions (mucositis, leukopenia, nausea & vomiting) than those receiving RT alone. By comparison however, in the EORTC 22931 trial Bernier et al showed that the incidence of late complications including muscular fibrosis, xerostomia (dry mouth), dysphagia (swallowing difficulty) and shoulder impairment were no different in patients treated with adjuvant radiotherapy or cisplatin based chemoradiotherapy (Bernier et al., 2004). Similar findings were seen in the in the Phase III GORTEC 94-01 trial, in which patients with OPSCC were treated with radiotherapy or chemoradiotherapy. In this study, there was no difference in long-term toxicities between the two treatment groups at five years (Denis et al., 2003). Although the EORTC & GORTEC studies show no significant increase in long-term toxicity with adjuvant chemoradiotherapy compared to radiotherapy alone, there is no evidence that chemoradiotherapy leads to improved quality of life outcomes. In addition there are a number of other reasons that the higher quality of life outcomes in the emotional and global domains of the MDADI in this study should be interpreted with caution. Firstly, the number of patients receiving adjuvant chemotherapy in this study is small (n=16), increasing the likelihood that this result may represent a chance finding. Furthermore, results from the UWQoL questionnaire do not support the

association between the higher scores in the global and emotional domains of the MDADI and treatment with adjuvant chemoradiotherapy (Table 18). In order to fully evaluate the impact of chemotherapy on long-term swallowing and quality of life outcomes, further large, prospective trials would be required.

3.4 Limitations of survival and functional outcome analysis

There are several limitations to the survival and quality of life analyses performed in this study. The study was performed in a single centre over a ten-year period. Although 168 patients were eligible for inclusion in the study, smaller numbers were included in the survival analysis (n=107) and quality of life analyses (n=72). Multicentre recruitment would increase the power of the analysis, with more events per variable, and would allow borderline associations to be more accurately defined. It would also allow the association between chemotherapy and improved functional outcomes to be explored in more detail.

Another limitation of the quality of life analysis is the retrospective collection of outcome data. Quality of life questionnaires were sent to patients a variable period (53 to 165 months) after completing treatment. Although time from treatment was not significantly associated with outcome in this study, the duration of time elapsed since treatment may introduce an element of recall bias. Prospective data collection pre-operatively and at defined post-operative intervals would define how quality of life outcomes vary with time and minimise recall bias.

Despite these limitations, this study provides valuable long-term outcome data in a specific subset of patients with HNSCC. The results show that current treatment regimens in OPSCC are associated with significant morbidity and mortality, and

indicate that new treatment approaches, which improve survival with low early and late toxicity, are urgently required.

Following encouraging translational and clinical studies, recent interest has focussed on the use of agents that target specific molecular pathways in HNSCC. EGFR inhibition is an established treatment in HNSCC, which potentiates the effect of radiotherapy and improves survival (Bonner et al., 2010, Clayburgh et al., 2013). Current trials are underway to investigate whether EGFR inhibition in combination with radiotherapy may also offer reduced treatment toxicity compared to chemoradiotherapy in OPSCC (PATHOS NCT02215265, RTOG-1016 NCT01302834). These trials highlight the significance of EGFR signalling in HNSCC, and support the investigation of other molecular pathways that may also play an important role in the development and progression of the disease. The following chapters will therefore assess the role of IGF-1R in HNSCC and evaluate the role of IGF-1R inhibition in this setting.

4 IGF-1R immunohistochemistry

4.1 Introduction

High IGF-1R expression has been shown to confer adverse prognosis in a range of tumour types (Turner et al., 1997b, Aleksic et al., 2010, Turney et al., 2011). An association between adverse survival and IGF-1R in HNSCC has also been reported (Lara et al., 2011). However, recent data highlighting the link between IGF-1R and known prognostic indicators in HSNCC draws the direct association between IGF-1R and survival into question (Matsumoto et al., 2014). This chapter will address the hypothesis that IGF-1R expression is associated with survival in HNSCC, and will aim to define the association between IGF-1R and clinical/pathological variables in patients with HSNCC and discuss the significance of these findings in the light of other recently published reports.

4.2 Optimisation of IGF-1R staining in HNSCC

Immunohistochemistry was performed according to the protocol previously established in our lab and described by Aleksic et al (Aleksic et al., 2010). Further optimisation was performed for HNSCC tissue, using whole-mount sections of tonsil squamous cell carcinoma, and cell pellets of SKUT-1 (IGF-1R deficient) and MCF-7 (strong IGF-1R expression) cells. To control for non-specific staining attributed to secondary antibody, no primary antibody was applied on one slide (Figure 5).

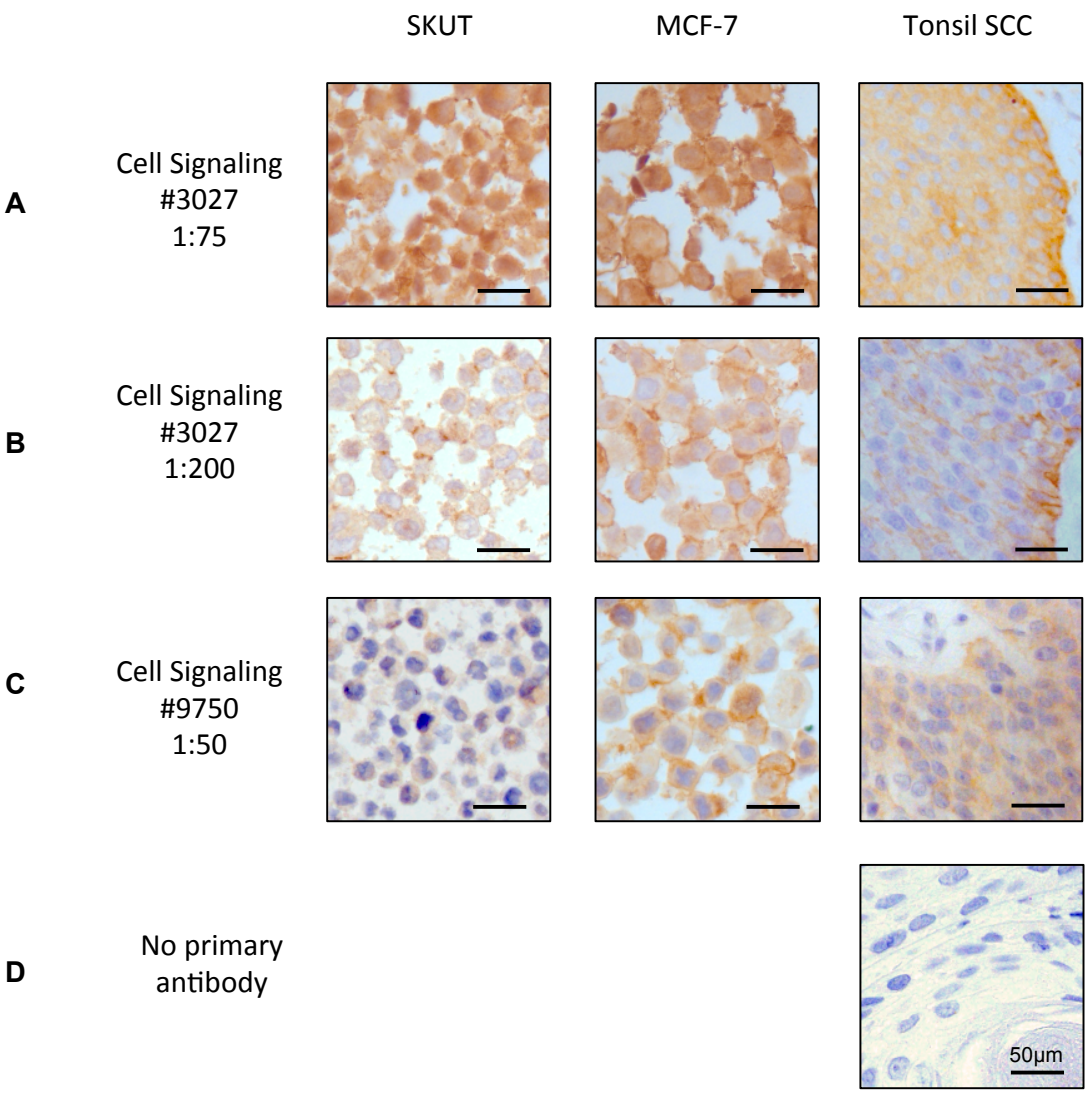


Figure 5: IGF-1R immunohistochemistry optimisation

A) Antibody #3027 (Cell Signaling) at 1:75 dilution, strong staining in all sections including IGF-1R deficient SKUT-1 cells.

B) Antibody #3027 (Cell Signaling) at 1:200 dilution, weak IGF-1R staining in IGF-1R deficient SKUT-1 cells.

C) Antibody #9750 (Cell Signaling) at 1:50 dilution, no staining in IGF-1R deficient SKUT-1 cells, with moderate intensity staining in positive control MCF-7 cells and tonsil SCC tissue.

D) Tonsil SCC control with no primary antibody.

All images are taken at 20x magnification.

Slides were reviewed with Consultant Head and Neck Pathologist Dr Ketan Shah, and final staining conditions selected. At both 1:75 and 1:200 dilutions of the #3027 polyclonal IGF-1R antibody (Cell Signaling), staining of the negative control SKUT-1 cells was observed. The #9750 monoclonal IGF-1R antibody (Cell Signaling) produced minimal staining in the SKUT-1 negative control cell pellet, with strong staining of the positive control MCF-7 cell pellet and the tonsil SCC, and was therefore selected for HNSCC TMA immunostaining.

4.3 HNSCC tissue microarray

Eight hundred and fifty two cores of HNSCC from 346 patients were included in the TMA. Tumours were from a variety of sub-sites within the head and neck: 67% Oropharynx (n=231), 24% Larynx (n=85), 8% Hypopharynx (n=28), 1% Oral cavity (n=2). Primary treatment modality was surgery in 88% (n=305), and radiotherapy or chemoradiotherapy in 12% (n=41), however the majority of patients received radiotherapy as part of their treatment (n=296, 86%). HPV status was determined by in-situ hybridisation for HPV DNA; 236 cases (68%) tested negative for HPV DNA and 110 (32%) were positive. Consistent with previous data, the proportion of HPV positive tumours varied by tumour sub-site (Leemans et al., 2011). HPV infection was detected in 50% of oropharyngeal tumours, 7% of hypopharyngeal tumours and 4% of laryngeal tumours. Pathological data were not available for all cases. In particular, the presence of extracapsular spread could only be determined in patients undergoing neck dissection for lymph node metastases and was found to be present in 102 of 200 cases (51%). In primary tumour cores, lymphovascular invasion was present in 79 of 270 primary tumours (29%), and perineural spread occurred in 66 of 270 (24%). Demographic data are shown in Table 19.

Demographic data		No. patients
Age	Mean 58.7 years	
	Range 19 – 86 years	
Gender	Male	264
	Female	82
Primary tumour site	Oropharynx	231
	Larynx	85
	Hypopharynx	28
	Oral cavity	2
Tumour T-stage	1	17
	2	40
	3	65
	4	219
HPV status	Negative	236
	Positive	110
Treatment	Primary surgery	305
	Radiotherapy	296
	Median dose 64 Gy, 32 fractions	
	Range 20 – 78 Gy, 5 – 38 fractions	
	Chemotherapy	49

Table 19: Demographic details for 346 patients included in HNSCC tissue microarray.

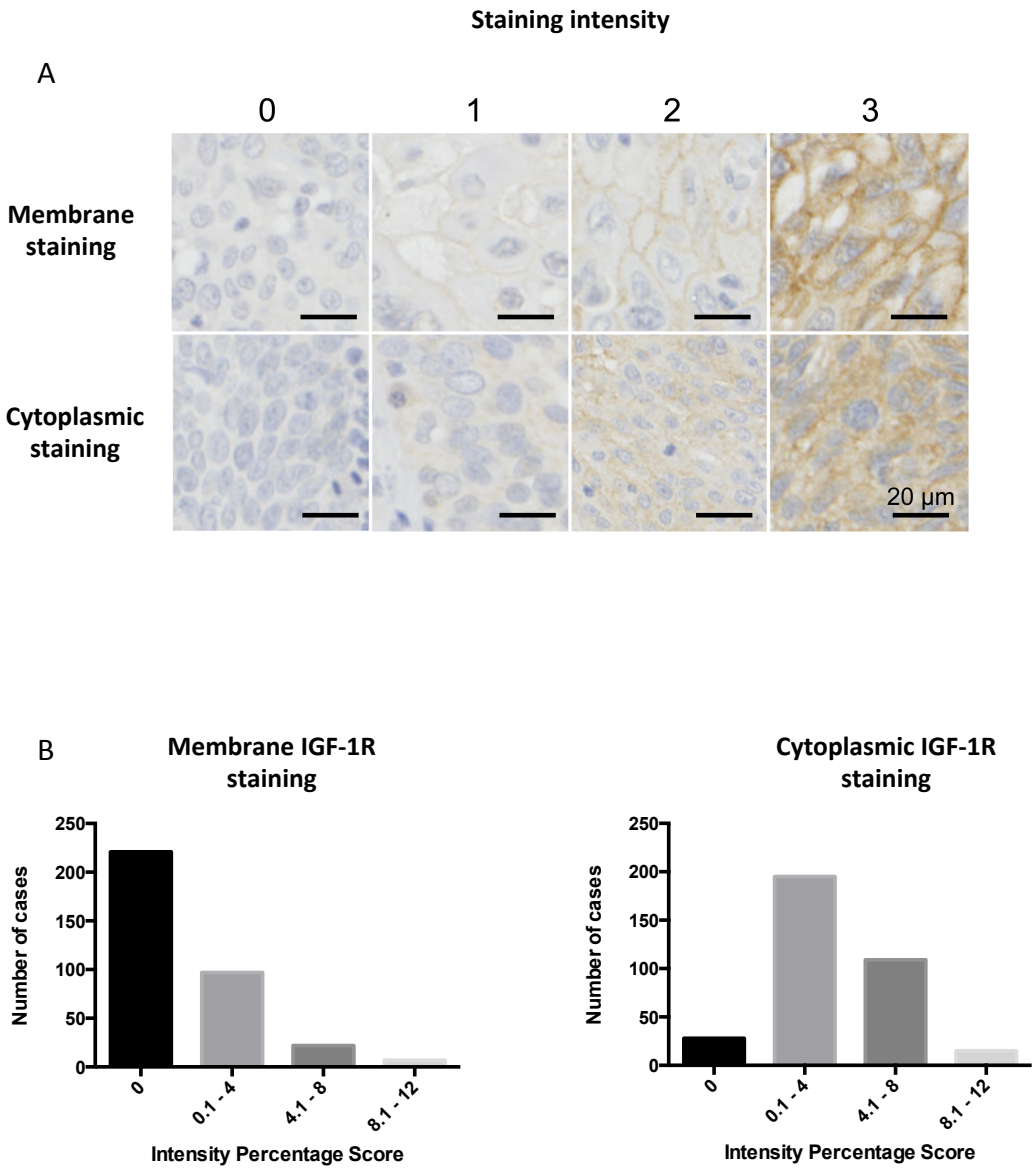


Figure 6: IGF-1R immunostaining of HNSCC tissue microarrays

A) Panels show representative tumour tissue from HNSCC TMAs stained with IGF-1R primary antibody #9750 (Cell Signaling). Intensity score of IGF-1R staining in the membranous and cytoplasmic compartments of HNSCC cells: 0 – no staining, 1 – weak, 2 – moderate, 3 – strong staining.

B) The distribution of IGF-1R staining intensity according to final IRS scores in both membranous and cytoplasmic compartments of HNSCC cells.

4.4 IGF-1R expression in HNSCC

Cores were scored for IGF-1R expression according to the intensity (0-3) and percentage (0-4) of tumour tissue stained. The product of these values gave the immunoreactive score (IRS) (Materials and Methods, Section 2.2.3). The intensity and the subcellular distribution of IGF-1R staining varied considerably between tumours; representative images of different staining patterns are shown in Figure 6A. In the majority of cases, cytoplasmic IGF-1R expression was low (IRS 0.1-4) and membrane IGF-1R expression was absent (IRS=0, Figure 6B). Linear regression analysis demonstrated a significant positive association between membrane and cytoplasmic IGF-1R expression ($p<0.001$, Figure 7A).

In 64 cases of oropharyngeal cancer, matched normal epithelial control tissue was available for analysis alongside HNSCC tumour tissue. Cytoplasmic IGF-1R expression was significantly higher in tumour tissue than in normal epithelial tissue ($p<0.001$, Figure 7B). Membrane IGF-1R expression was not detected in any of the normal tissue specimens analysed. Despite the majority of tumours in this TMA demonstrating weak cytoplasmic and absent membranous IGF-1R expression, these data suggest that IGF-1R is overexpressed in HNSCC relative to matched normal control tissue.

These results support the findings of previous studies. In 2007, Barnes et al. prepared cell lysates from 12 oral cavity SCCs and matched normal tissues and quantified IGF-1R expression by western blotting, showing increased IGF-1R expression in tumour tissues compared to normal tissues. Similar results were obtained in HNSCC cell lines, with higher IGF-1R levels than keratinocyte controls (Barnes et al., 2007). Immunostaining for IGF-1R in HNSCC has been reported in several studies. Rezec et al. examined a series of 57 oral cavity SCCs and found

that 56% expressed IGF-1R, although no comparison to normal epithelial tissue was made (Reszec et al., 2004). Lara et al. performed IGF-1R immunostaining on a series of 131 patients with oral cavity SCC, and found that only 23% showed no IGF-1R expression. In that study, the majority of tumours (46%) exhibited moderate or high IGF-1R staining, compared to 36% in the current study. This variation may be due to differences in immunostaining conditions; in the study by Lara et al, an antibody directed against the α-subunit of IGF-1R was used. Data from the current study demonstrate how immunostaining results may differ depending on the staining conditions and the primary antibody (Results section 4.2).

Having identified that IGF-1R is overexpressed in HNSCC, and that the extent of IGF-1R expression varies between tumours, data were analysed to investigate the relationship between IGF-1R and clinical variables.

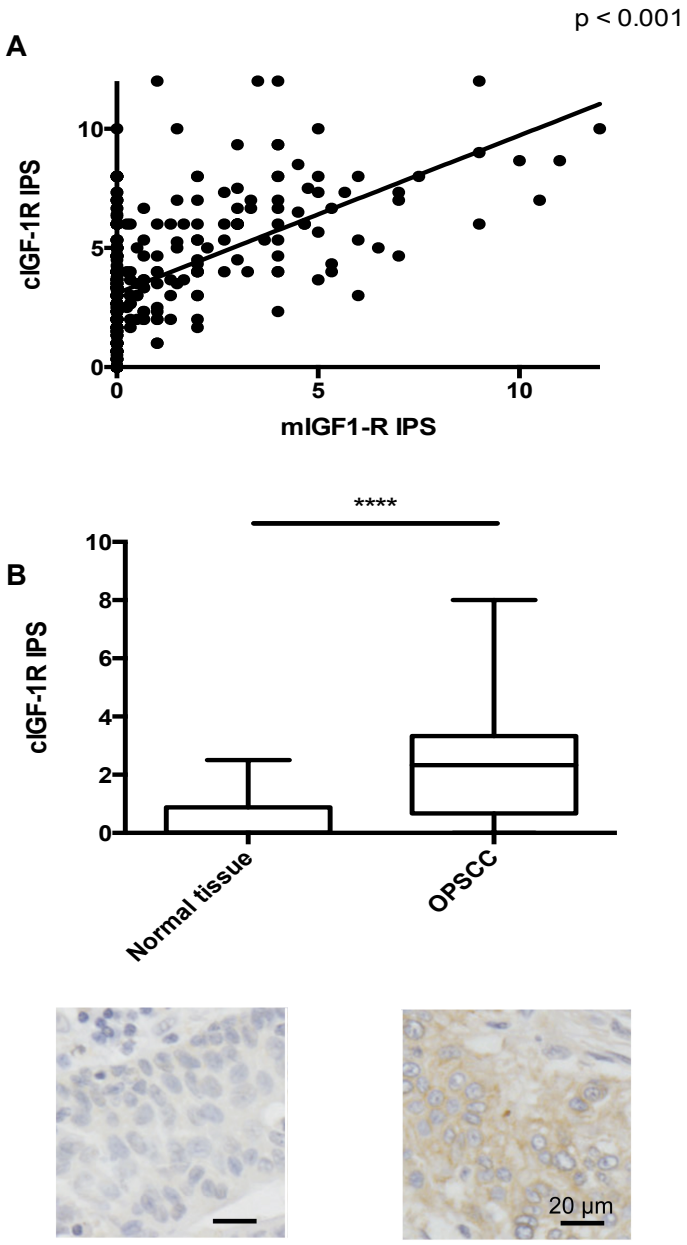


Figure 7: IGF-1R expression in HNSCC

A) Linear regression analysis of mean cytoplasmic IGF-1R IRS (cIGF-1R) and mean membrane IGF-1R IRS (mIGF-1R) in 346 cases of HNSCC. A strong correlation was observed between cIGF-1R and mIGF-1R expression ($p<0.001$).
B) Cytoplasmic IGF-1R IRS (cIGF-1R) in HNSCC (right) and matched epithelial control tissue (left), showing significantly greater expression of IGF-1R in HNSCC compared to normal tissue ($P<0.001$, paired T test).

4.5 IGF-1R expression and survival in HNSCC – Univariate analysis

In order to identify associations between IGF-1R expression and clinical variables in HNSCC, an initial univariate analysis was performed. For the purposes of analysis, IGF-1R IRS data were grouped into categorical variables. Due to the high number of tumours that lacked detectable membrane IGF-1R, membrane IGF-1R IRS data were grouped into tumours with absent membrane IGF-1R (IRS = 0, n=220), and those with membrane IGF-1R expression (IRS > 0, n=126). For cytoplasmic IGF-1R expression, data were grouped into tumours with absent or low cytoplasmic IGF-1R expression (0≤IRS≤4, n=182), and those expressing moderate to high IGF-1R (4<IRS≤12, n=164). Total IGF-1R expression (the sum of membrane plus cytoplasmic IRS) was categorised as low (cIGF-1R + mIGF-1R ≤ 4.5) or moderate to high (cIGF-1R + mIGF-1R > 4.5).

The results of univariate analysis suggested that high membrane, cytoplasmic and total IGF-1R expression were associated with reduced overall and disease specific survival (Table 20). These findings were supported by Kaplan-Meier analysis, which showed that high IGF-1R expression was associated with reduced survival at 5 years (Figure 8).

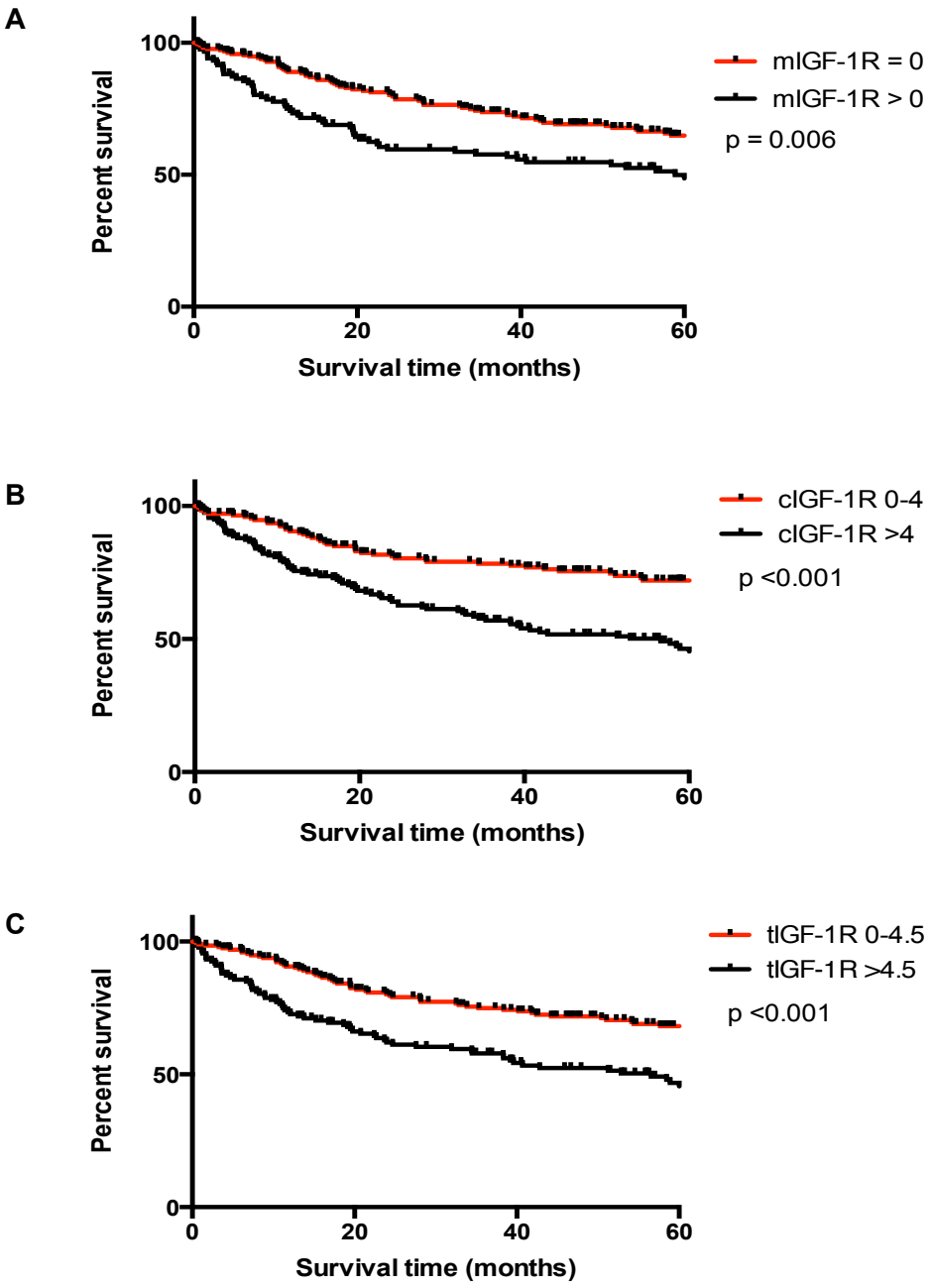


Figure 8: Kaplan-Meier analysis showing the relationship between IGF-1R expression and overall survival in HNSCC.

A) Reduced overall survival in patients with tumours expressing membrane IGF-1R (mIGF-1R) (IRS > 0) compared to those with no detectable mIGF-1R staining (IRS = 0).
B) Reduced overall survival in patients with moderate to high tumoural cytoplasmic IGF-1R (cIGF-1R) expression (IRS > 4), compared to those with low or absent cIGF-1R expression (IRS 0-4).
C) Reduced overall survival in patients whose tumours express higher total IGF-1R (tIGF-1R = mIGF-1R + cIGF-1R) (tIGF-1R > 4.5) compared to those with low t-IGF-1R expression (IRS 0-4.5).

A: Overall survival			
Variable	**HR**	**95% CI**	**P value**
Age	1.05	1.03 – 1.07	< 0.001
HPV positive status	0.37	0.23 – 0.59	< 0.001
Tumour 'T' stage	1.13	0.91 – 1.39	0.274
Lymphovascular invasion	2.12	1.41 – 3.20	< 0.001
Perineural invasion	2.15	1.41 – 3.28	< 0.001
Extracapsular spread	1.36	0.86 – 2.16	0.186
Cytoplasmic IGF-1R	2.17	1.52 – 3.10	< 0.001
Membrane IGF-1R	1.63	1.16 – 2.30	0.006
Total IGF-1R	2.00	1.41 – 2.82	< 0.001
B: Disease specific survival			
Variable	**HR**	**95% CI**	**P value**
Age	1.05	1.03 – 1.07	< 0.001
HPV positive status	0.35	0.20 – 0.60	<0.001
Tumour 'T' stage	1.36	1.03 – 1.79	0.032
Lymphovascular invasion	3.05	1.91 – 4.87	< 0.001
Perineural invasion	3.11	1.95 – 4.98	< 0.001
Extracapsular spread	1.86	1.08 – 3.21	0.026
Cytoplasmic IGF-1R	1.91	1.27 – 2.86	0.002
Membrane IGF-1R	1.63	1.10 – 2.43	0.016
Total IGF-1R	2.16	1.45 – 3.21	<0.001

Table 20: Univariate analysis of clinical and pathological variables in HNSCC

The effect of clinical and pathological variables, including cytoplasmic (IRS <4 vs ≥4), membrane (IRS 0 vs >0) and total (IRS ≤4.5 vs >4.5) IGF-1R scoring, on A) overall survival, B) disease specific survival of patients with HNSCC, showing Hazard Ratio (HR) for death, with 95% confidence intervals (CI).

Univariate analysis of clinical and pathological variables also demonstrated other associations with survival. Increasing age, perineural spread and lymphovascular invasion were associated with adverse overall and disease specific survival, while HPV positive status was associated with favourable survival outcomes. The presence of extracapsular spread and tumour T stage were associated with reduced disease specific survival but did not affect overall survival. (Table 20 & Figure 9 A-D).

The results from univariate analysis suggested that higher membrane, cytoplasmic and total IGF-1R expression may be associated with adverse overall and disease specific survival in HNSCC. However, this analysis does not control for other significant prognostic variables, which may influence IGF-1R expression and survival in this group of patients. High tumour T stage (a surrogate marker for tumour size), the presence of perineural spread and lymphovascular invasion in the primary tumour, extracapsular spread in lymph node metastases and HPV status are all known to be of prognostic significance in HNSCC (Argiris et al., 2008, Ang et al., 2010). Univariate and subsequent Kaplan-Meier analysis confirmed the significance of these variables on overall survival, suggesting that this cohort of patients was representative of HNSCC cohorts described previously (Figure 9) (Ang et al., 2010).

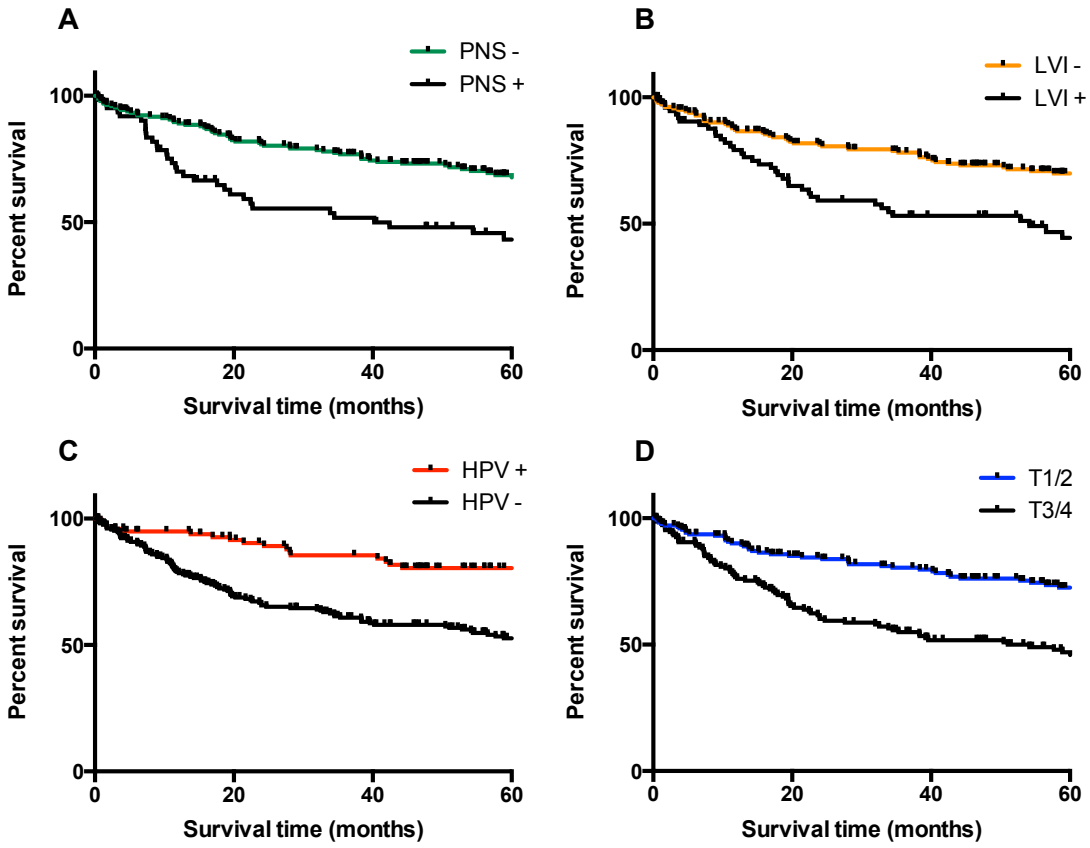


Figure 9: The effect of known prognostic variables on overall survival in HNSCC.

A) The presence of perineural spread (PNS) in primary HNSCC tumours was associated with reduced overall survival at 5 years ($p<0.001$).
B) The presence of lymphovascular invasion (LVI) in primary HNSCC tumours was associated with reduced overall survival at 5 years ($p<0.001$).
C) HPV negative status was associated with reduced overall survival at 5 years compared to HPV positive disease ($p<0.001$)
D) Smaller tumours (T stage 1/2) were associated with improved overall survival at 5 years compared to larger tumours (T stage 3/4) ($p<0.001$).

The next analyses tested associations between IGF-1R and other clinical variables. Higher expression of membrane, cytoplasmic and total IGF-1R were significantly associated with HPV negative status (Figure 10 A). High membrane, cytoplasmic and total IGF-1R expression were also associated with increased tumour T stage (Figure 10 B), but not significantly associated with perineural spread, lymphovascular invasion or extracapsular spread. Finally, HPV status was significantly related to tumour T stage: HPV negative disease was associated with a higher tumour T stage ($p<0.001$).

The significant association between HPV status and IGF-1R expression identified in the present large cohort supports the recent findings of Matsumoto and colleagues (Matsumoto et al., 2014). In a study of 59 patients with oropharyngeal cancer the authors found that HPV negative status was significantly associated with high IGF-1R expression, and that patients with high tumoural IGF-1R expression had poorer disease specific survival than those with low IGF-1R expression. In the same study, however, no significant association was observed between IGF-1R and T stage, although there was a trend towards higher IGF-1R expression in larger tumours ($p=0.087$).

Analysis of IGF-1R expression by anatomical sub-site showed that IGF-1R expression was significantly lower in oropharyngeal cancers compared to cancers of the larynx or hypopharynx ($P<0.001$). This is likely to reflect the higher proportion of HPV positive tumours in the oropharynx.

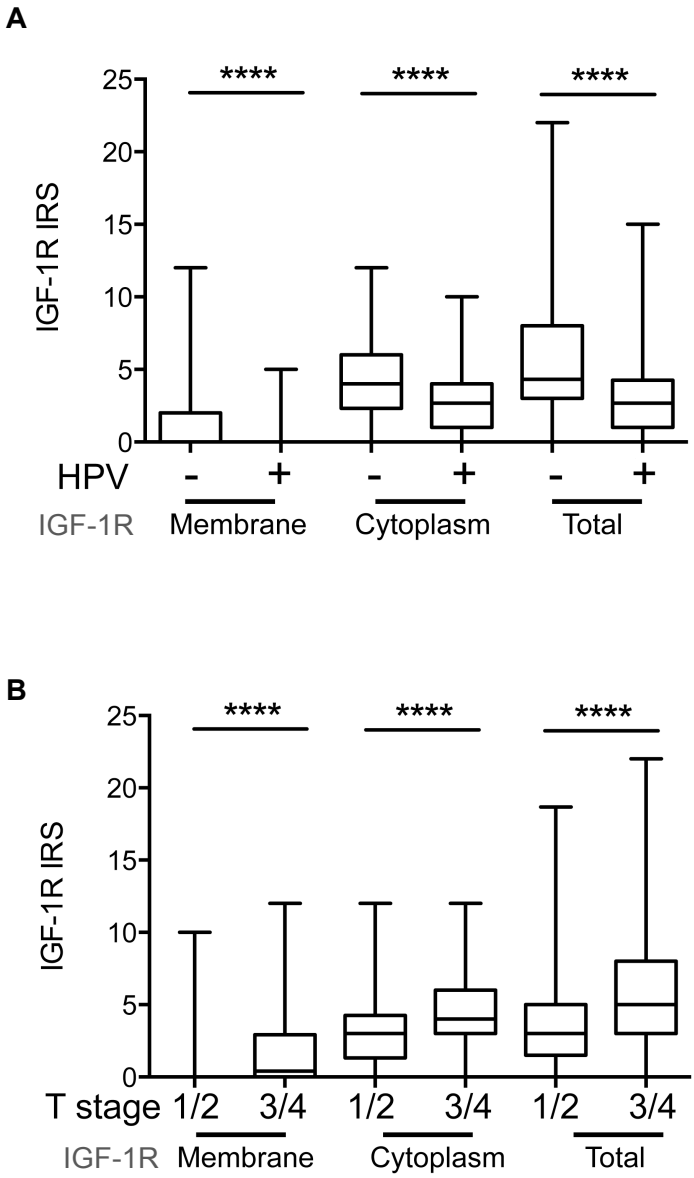


Figure 10: The association between IGF-1R expression and HPV status/tumour 'T' stage in HNSCC

A) Tumours testing negative for HPV DNA on in-situ hybridisation were found to have increased membrane (mIGF-1R), cytoplasmic (cIGF-1R) and total (tIGF-1R) IGF-1R expression (Mann-Whitney U Test, **** p<0.001). B) Tumours with higher 'T' stage (T3/T4) contain higher levels of membrane, cytoplasmic and total IGF-1R than tumours with lower 'T' stage (T1/2) (****P<0.001, Mann-Whitney U Test).

The relationship between HPV status and T stage was defined by Ang et al in 2010 in a large study of 721 patients with HNSCC. HPV positive disease was associated with lower tumour T stage and also several other good prognostic factors including non-smoking status, improved performance status and younger age at diagnosis (Ang et al., 2010).

These data and results from the current study provide evidence for the complex interaction between HPV status, tumour T stage and IGF-1R expression, in which higher IGF-1R expression is associated with HPV negative status and increased tumour T stage, and where T stage and HPV status are also directly related. Given the close relationship of these variables, it is not possible to determine from univariate analysis whether IGF-1R expression is independently associated with survival in HNSCC. For that reason, a multivariate analysis was performed.

4.6 IGF-1R expression and survival in HNSCC – Multivariate analysis

Significant associations from univariate analysis were entered into multivariate analysis to determine whether variables were independently associated with overall survival. For the purposes of multivariate analysis, total IGF-1R was used in preference to cytoplasmic or membranous IGF-1R, since it demonstrated the largest effect on disease specific survival in univariate analysis (HR total IGF-1R = 2.16, HR membranous IGF-1R = 1.63, HR cytoplasmic IGF-1R = 1.91).

Due to the significant association between HPV status and IGF-1R expression, these variables alone were entered into multivariate analysis, to determine whether the effect on survival of both variables was independent of each other. This suggested that high IGF-1R expression and HPV negative status were independently associated with adverse overall survival in HNSCC (Table 21 A). These findings were supported by Kaplan Meier analysis, which showed that high IGF-1R expression was associated with reduced overall survival in both HPV positive and HPV negative subgroups (Figure 11).

In order to control for other variables that may independently influence survival, a further multivariate analysis was performed. This included the significant pathological prognostic indicators that were identified in the initial univariate analysis. HPV status, lymphovascular invasion, perineural spread and tumour T stage, were confirmed to be independent prognostic indicators of overall survival in HNSCC. Total IGF-1R expression however, appeared not to be an independent predictor of survival in this cohort (Table 21 B).

A			
Variable	**HR**	**95% CI**	**P value**
HPV positive status	0.42	0.26 – 0.67	<0.001
Total IGF-1R	1.74	1.22 – 2.46	0.002
B			
Variable	**HR**	**95% CI**	**P value**
Lymphovascular invasion	1.76	1.15 – 2.68	0.009
Perineural spread	1.62	1.05 – 2.50	0.030
Tumour 'T' stage	1.60	1.02 – 2.50	0.042
HPV positive status	0.46	0.26 – 0.84	0.011
Total IGF-1R	1.30	0.84 – 2.01	0.246
C: HPV negative cases			
Variable	**HR**	**95% CI**	**P value**
Lymphovascular invasion	1.87	1.19 – 2.95	0.007
Perineural spread	1.72	1.09 – 2.73	0.021
Tumour 'T' stage	1.75	1.06 – 2.89	0.029
Total IGF-1R	1.08	0.68 – 1.72	0.737
D: HPV positive cases			
Variable	**HR**	**95% CI**	**P value**
Lymphovascular invasion	1.65	0.48 – 5.69	0.428
Perineural spread	1.11	0.21 – 5.79	0.901
Tumour 'T' stage	1.11	0.31 – 3.93	0.872
Total IGF-1R	2.59	0.85 – 7.87	0.093

Table 21: Multivariate analysis of clinical and pathological parameters in HNSCC

A) The effect of total IGF-1R expression (IRS ≤4.5 vs >4.5) and HPV status (HPV positive vs HPV negative) on overall survival in HNSCC (n=338), showing hazard ratio (HR) for death and 95% confidence intervals (CI). **B**) The effect of other prognostic indicators, including tumor T-stage (1-2 vs 3-4) on the significance tIGF-1R expression and HPV status as predictors of overall survival in HNSCC (n=262). **C, D)** The effect of clinical and pathological variables including IGF-1R (total IRS ≤4.5 vs >4.5) on overall survival in **C)** HPV negative patients (n=176) and **D)** HPV positive patients (n=86).

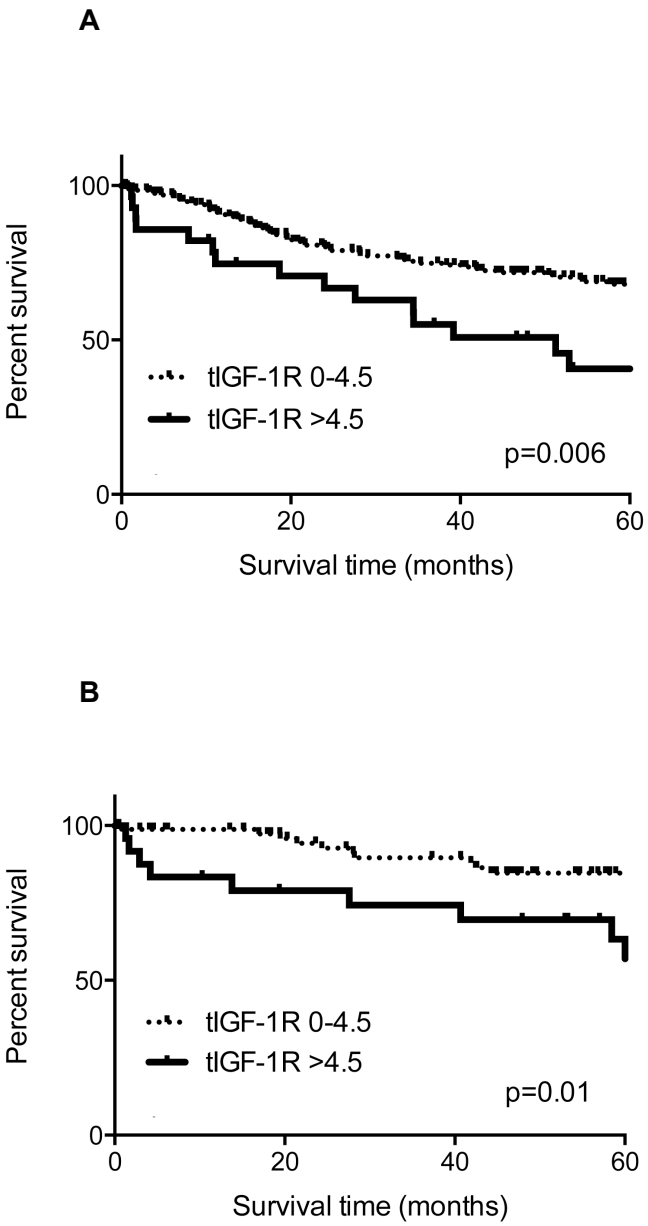


Figure 11: Kaplan Meier analysis showing the effect of total IGF-1R expression on overall survival in HPV positive and HPV negative HNSCC.

Higher total IGF-1R expression (IRS > 4.5) is associated with reduced overall survival in patients with both HPV negative (A) and HPV positive (B) HNSCC.

In order to further investigate the relationship between IGF-1R expression and HPV status, patients were stratified by HPV status and a further multivariate analysis performed (Table 21 C & D). Subgroup analysis showed that lymphovascular invasion, perineural spread and tumour T stage were of prognostic significance in patients with HPV negative tumours (n=176, 81 deaths), but total IGF-1R expression showed no significant relationship with overall survival (Table 21 C). The group of patients with HPV positive tumours was smaller (n=86, 15 deaths), with fewer events, making it more difficult to draw firm conclusions from these data. However, none of the variables analysed showed a significant association with overall survival (Table 21 D).

4.7 Discussion

An association between adverse survival in HNSCC and high IGF-1R expression was first identified by Lara et al in 2011. They found that IGF-1R expression was not predictive of survival in a cohort of 131 cases of predominantly oral cavity SCC, but subgroup analysis showed that patients with a high tumour T stage and high IGF-1R expression had poorer survival outcomes at 5 years than those with high T stage and low IGF-1R expression (Lara et al., 2011). Although the authors used multivariate analysis to assess the influence of other predictors of survival in this study, HPV data were not presented, which could introduce an important confounding variable. Additional evidence for the relationship between IGF-1R expression and survival in HNSCC was provided by Mountzios et al. who showed reduced overall survival in patients with laryngeal cancer and high membrane or cytoplasmic IGF-1R immunostaining (Mountzios et al., 2013). Again, HPV data were not reported, however, the inclusion of only patients with laryngeal cancer is likely to reduce the proportion of HPV positive cases, and therefore reduce the influence of HPV as a

confounding variable. Interestingly, in both of these studies an antibody against the α-subunit of the IGF-1R was used. Moreover, immunostaining with an antibody against the β-subunit of the IGF-1R showed no relationship with survival (Mountzios et al., 2013).

Sun et al. showed that other elements of the IGF axis may be important in predicting survival in HNSCC. In a study of 131 patients undergoing surgery for HNSCC, they found that IGFBP3 expression was associated with a shorter time to progression (TTP), but was not independently linked with overall survival in multivariate analysis (Sun et al., 2011). Although IGF-1R expression itself was not related to overall survival or TTP in that study, the authors found that the co-expression of the receptor with IGFBP3 in HNSCC tissue was associated with a significantly shorter TTP. This may relate to the effect of IGFBP3 on IGF-1R signalling. Although generally regarded as an IGF antagonist (Franklin et al., 2003), cell-associated IGFBP3 has been shown to potentiate IGF-1R signalling by modulating the PI3K pathway; increasing the sensitivity of PKB/AKT to IGF ligand stimulation (Conover et al., 2000).

A possible shortcoming in some of the existing reports of IGF-1R in HNSCC is the failure to control for known prognostic indicators, in particular HPV status (Sun et al., 2011, Lara et al., 2011, Mountzios et al., 2013). Data from this study, confirm previous reports that HPV status, lymphovascular invasion, perineural spread and tumour T stage are important prognostic indicators (Table 21) (Ang et al., 2010, Fagan et al., 1998, Woolgar, 2006). Indeed, HPV status has been deemed so significant in HNSCC, that in their review of the molecular biology of HNSCC, Leemans et al stated that studies using survival as an outcome measure should always include HPV status in multivariate analysis, as it an important potential confounding factor (Leemans et al., 2011). In the current setting, this statement is particularly pertinent, given the association observed between HPV status and IGF-1R expression.

After controlling for HPV status and other pathological variables, data from this study indicate that IGF-1R expression was not independently associated with survival outcome in HNSCC. An association between IGF-1R expression and survival has, however, been demonstrated in other solid tumour types (Hirakawa et al., 2013, Turney et al., 2011, Turner et al., 1997b). In addition, work from this group has shown that not only expression levels, but also sub-cellular localisation of the IGF-1R is of prognostic significance. Aleksic et al. showed that IGF-1R can undergo nuclear import, and that nuclear IGF-1R is associated with an adverse prognosis in clear cell renal cancer. Furthermore IGF-1R undergoes phosphorylation in response to IGF ligand, leading to an interaction between IGF-1R and chromatin (Aleksic et al., 2010). This suggests a role for nuclear IGF-1R in transcriptional regulation, and highlights the significance of the subcellular localisation of IGF-1R. In the current study nuclear IGF-1R was not detectable in HNSCC, although a significant proportion of tumours analysed expressed membrane IGF-1R (36%). The significance of membrane IGF-1R is uncertain. In cell line models, serum starvation leads to increased membrane IGF-1R expression. Subsequent application of IGF-1 ligand leads to internalisation and degradation of the receptor or recycling back to the cell surface (Aleksic et al., 2010, Romanelli et al., 2007). This could suggest that membrane IGF-1R implies inactive receptor, while cytoplasmic (internalised) receptor implies activation. However, results from this study reveal a close correlation between membrane-bound and cytoplasmic IGF-1R expression (Figure 7A), and increased membrane IGF-R expression may simply reflect upregulation of the IGF axis as a whole in some HNSCCs. Although not independently related to survival outcome following multivariate analysis, the association of both membrane and cytoplasmic IGF-1R with HPV negative disease and higher tumour T stage, indicate that IGF-1R overexpression, regardless of subcellular localisation, is associated with a more aggressive disease phenotype (Figure 10). Further support for this comes from studies, which suggest that IGF-1R expression is associated with higher tumour

grade in HNSCC and pancreatic cancer (Hirakawa et al., 2013, Lara et al., 2011). Data on tumour grade were not available in this study, therefore assessment of correlation with IGF-1R expression was not possible.

The results presented in this study suggest that HPV infection is associated with reduced IGF-1R expression in HNSCC (Figure 10), supporting recently published data in smaller series (Matsumoto et al., 2014, Oh et al., 2013). Other studies have shown that EGFR expression is affected in a similar way: EGFR expression is significantly lower in HPV positive HNSCC compared to HPV negative disease (Husain et al., 2012, Burtness et al., 2013). The unique tumour biology of HPV positive HNSCC has been well described; the HPV viral oncoproteins E6 and E7 inactivate the tumour suppressor proteins p53 and Retinoblastoma (Rb) respectively, causing disruption of cell cycle regulation (Leemans et al., 2011). How these changes relate to IGF-1R and EGFR expression and the potential contribution of these changes to tumour phenotype remain to be defined. Investigation of this link could provide a particularly interesting avenue for further study because in the majority of cases, oncogenic transformation is association with EGFR and IGF-1R up-regulation, rather than down-regulation (Zhao et al., 2010, Weisberg et al., 2014).

Data from this study suggest that overexpression of IGF-1R in HNSCC is not independently associated with reduced survival, but that it is associated with an aggressive disease phenotype. This renders IGF-1R an attractive therapeutic target. The following chapter will address targeting the IGF-1R in HNSCC.

5 Targeting the IGF-1R in HNSCC cell lines

5.1 Introduction

The results from Chapter 4 indicate that IGF-1R is overexpressed in HNSCC and that higher levels of IGF-1R are associated with adverse clinico-pathological features including HPV negative status and advanced T stage. The mechanism of IGF-1R overexpression in HNSCC is not fully understood, and its association with adverse prognostic indicators does not necessarily imply causality. These findings do however suggest that the investigation of IGF-1R as a therapeutic target in HNSCC is warranted.

In spite of promising preclinical data (Riesterer et al., 2011b, Barnes et al., 2007), IGF-1R monotherapy has shown limited efficacy in unselected palliative patients with HNSCC (Schmitz et al., 2012). This suggests that biomarkers indicating sensitivity to IGF-1R inhibition are urgently required. This chapter will address the hypothesis that IGF-1R inhibition reduces cell survival in HNSCC cells. It will also test for correlations between sensitivity to IGF-1R inhibition and the expression of IGF-1R axis components to examine that hypothesis that these may act as a biomarker for sensitivity or resistance to IGF-1R inhibition.

5.2 IGF-1R signalling in HNSCC cell lines

In order to investigate the significance of IGF-1R signalling in HNSCC, expression of IGF-1R and related signalling proteins was determined in a panel of HNSCC cell lines by western blotting. The cell line panel consisted of seven HNSCC cell lines: four derived from anterior tongue SCCs (SAS, CAL-27, BICR-56, SCC-9) and three

from SCCs of the oropharynx (UM-SCC-50, UM-SCC-99, UT-SCC-60A). All cell lines were of known HPV negative status. The cell lines MCF-7 (breast cancer) and SKUT-1 (leiomyosarcoma) were used as positive and negative controls for IGF-1R. Using a polyclonal antibody against the β-subunit of the IGF-1R (Cell Signaling #3027), IGF-1R was not detectable in negative control SKUT-1 cells, but was strongly expressed in the positive control MCF-7 cells.

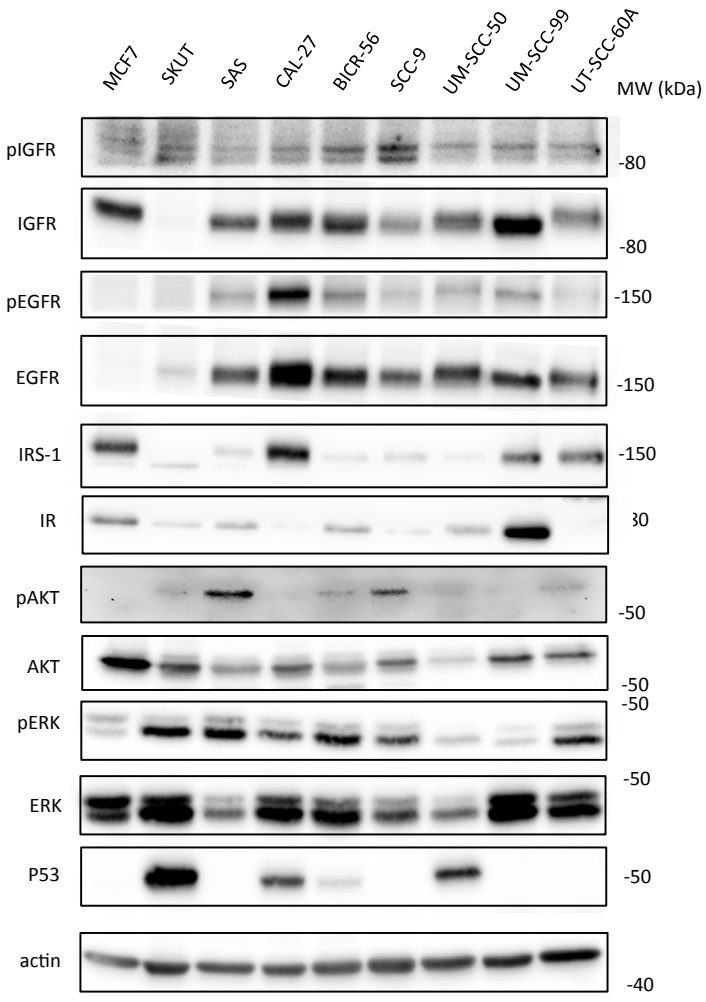


Figure 12: Expression of IGF axis components in HNSCC cell lines

HNSCC cell lines (SAS, CAL-27, BICR-56, SCC-9, UM-SCC-50, UM-SCC-99, UT-SCC-60A) and IGF-1R positive (MCF-7) and deficient (SKUT-1) controls. Cells were cultured in DMEM + 10% FCS at 10% CO_2. Lysates were prepared in RIPA lysis buffer and analysed by western blotting. Representative results are shown for 2-3 independent sets of lysates.

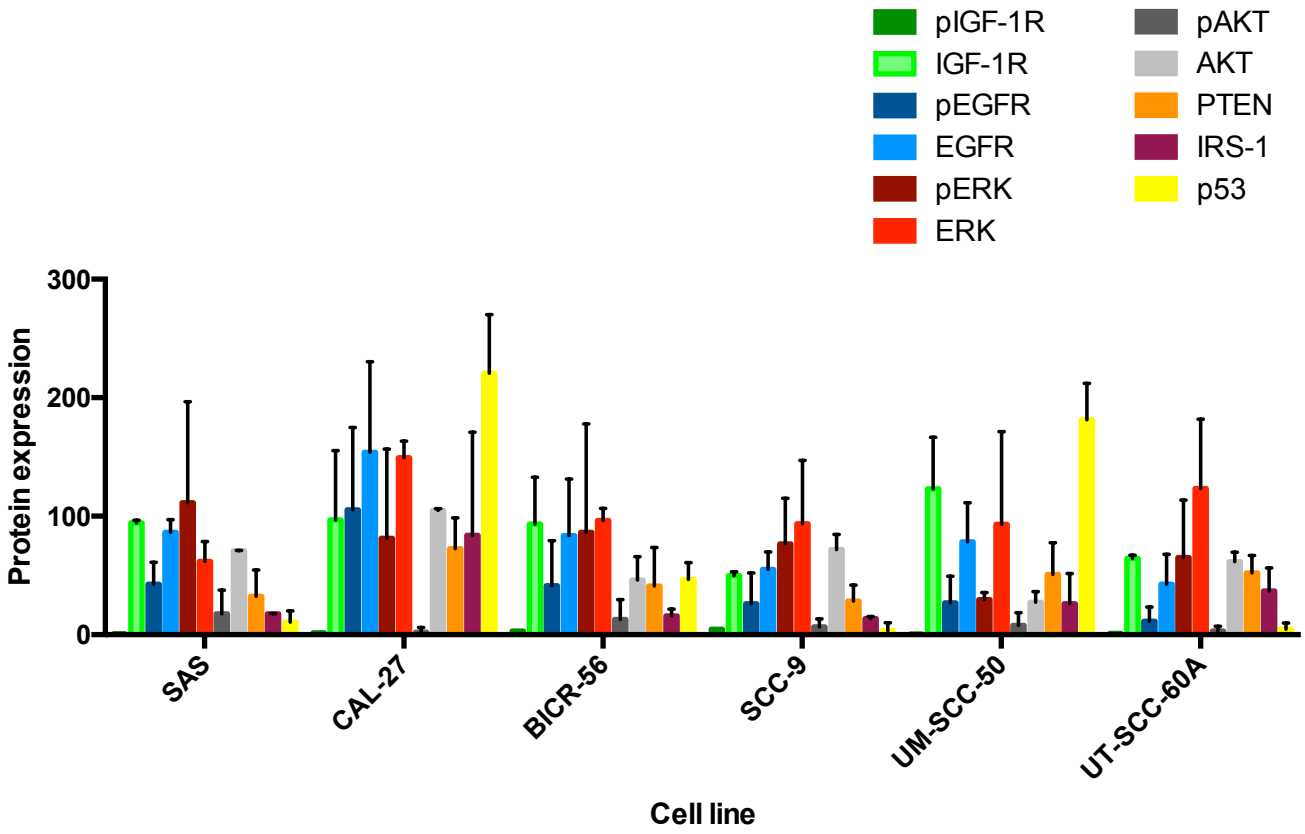


Figure 13: Quantification of protein expression in HNSCC cell lines.

Protein expression in HNSCC cell lines was determined by western blotting (Figure 12) and quantified using image densitometry (ImageJ, Bethesda, USA). Values represent the relative density of protein bands following subtraction of background signal and adjustment for loading. Representative results are shown for 2-3 independent sets of lysates.

IGF-1R expression was detected in all HNSCC cell lines tested, a finding that supports the data from the previous chapter. Although detected in all seven HNSCC cell lines however, IGF-1R expression levels varied. After controlling for loading, IGF-1R expression was highest in UM-SCC-99 cells, and lowest in SCC-9 cells (Figure 12) although this did not translate to higher levels of IGF-1R phosphorylation; SCC-9 cells showed the strongest phosphorylated IGF-1R signal despite having the lowest expression of total IGF-1R. Overall however, expression of phosphorylated IGF-1R was weak in all HNSCC cell lines tested. There are several possible explanations for this. Firstly, the HNSCC cell lines tested may not be strongly dependent on the IGF-1R axis for growth and proliferation, resulting in low levels of basal IGF-1R

phosphorylation. Previous studies however have demonstrated that inhibition of IGF-1R in HNSCC cell lines results in reduced cell survival (Barnes et al., 2007), suggesting that HNSCC cells are indeed dependent upon IGF-1R signalling for proliferation and survival. A second explanation is that IGF-1R activation is known to be tightly regulated (Pollak, 2012), and only low level IGF-1R phosphorylation is required for activation of downstream signalling pathways. Support for this hypothesis is derived from the phosphorylation of AKT observed in the BICR-56 and SAS cell lines that demonstrate weak IGF-1R phosphorylation. Finally, cell lysates for the western blots shown in Figure 12 were prepared from cells growing in DMEM with 10% FCS, without exogenous IGF-1 ligand stimulation. The relatively low levels of IGF-1 ligand in 10% FCS may explain the weak pIGF-1R signal observed in this panel.

All HNSCC cell lines in this panel also demonstrated EGFR expression, a finding that mirrors clinical data suggesting that EGFR is expressed in up to 90% of HNSCCs (Licitra et al., 2011, Dequanter et al., 2012). The level of EGFR expressed in this cell panel varied between cell lines. The CAL-27 cell line demonstrated the highest level of EGFR expression and phosphorylation, which was associated with phosphorylation of ERK, but not of AKT. The levels of other proteins involved in IGF-1R signalling including insulin receptor, IRS-1 and p53 also varied. The molecular diversity of HNSCC has been previously described by Wu et al. following proteomic analysis of 34 HNSCC cell lines. They found significant differences in the levels of 42 protein kinases involved in cell survival and proliferation pathways, some of which predicted sensitivity to kinase inhibition (Wu et al., 2011). IGF-1R, however, was not included in this study.

5.3 Inhibition of IGF-1R in HNSCC cell lines

In light of the data presented in Chapter 4 indicating that IGF-1R is overexpressed and associated with an aggressive disease phenotype in HNSCC, it is possible that the IGF-1R axis may play a significant role in HNSCC. In order to investigate the effect of IGF-1R on cell signalling and cell survival in HNSCC, IGF-1R was inhibited using the tyrosine kinase inhibitors AZ12253801 and BMS-754807. Previous data from our group indicate that AZ12253801 inhibits IGF-1R phosphorylation and downstream activation of AKT and ERK in prostate cancer cells and also reduces cell survival in a dose dependent manner (Chitnis et al., 2013). Similar results have been observed by another group using the BMS-754807 compound in a range of tumour types, including HNSCC (Carboni et al., 2009). BMS-754807 was found to reduce cellular proliferation in HNSCC, with an IC_{50} of 0.7-4.5μM. The sensitivity of other cancer cell lines to BMS-754807 varied, with IC_{50} values of 0.01-1.7μM in Ewing's sarcoma and 0.4->5μM in pancreatic cancer cell lines.

In this study, both AZ12253801 and BMS-754807 caused a reduction in IGF-1R phosphorylation in SAS cells (Figure 14A & Figure 15A). AZ12253801 caused a dose dependent reduction in both IGF-1R and AKT phosphorylation, with complete abrogation of pIGF-1R and pAKT signal at a concentration of 100nM (Figure 14A). In contrast, ERK phosphorylation was not inhibited, with evidence of persisting ERK phosphorylation at both 100nM and 300nM. In clonogenic survival assay, AZ12253801 caused a dose dependent reduction in cell survival, with an SF_{50} of 80nM (Figure 14B).

BMS-754807 inhibited IGF-1R phosphorylation at 10nM, and caused a dose dependent reduction in both AKT and ERK phosphorylation. At a concentration of 30nM BMS-754807, pAKT signal was reduced to that of the non-stimulated control,

however, weak ERK phosphorylation was still detectable at a concentration of 3uM. These experiments indicate that AZ12253801 and BMS-754807 have similar effects on IGF-1R and AKT phosphorylation. The effect on ERK phosphorylation however, differs. BMS-754807 causes greater reduction of ERK phosphorylation than AZ12253801. By contrast, BMS-754807 causes less potent inhibition of cell survival than AZ12253801 (Table 22). The cause of this variation is unclear, but may relate to the off target effects of these agents, which may inhibit other intracellular kinases (Carboni et al., 2009).

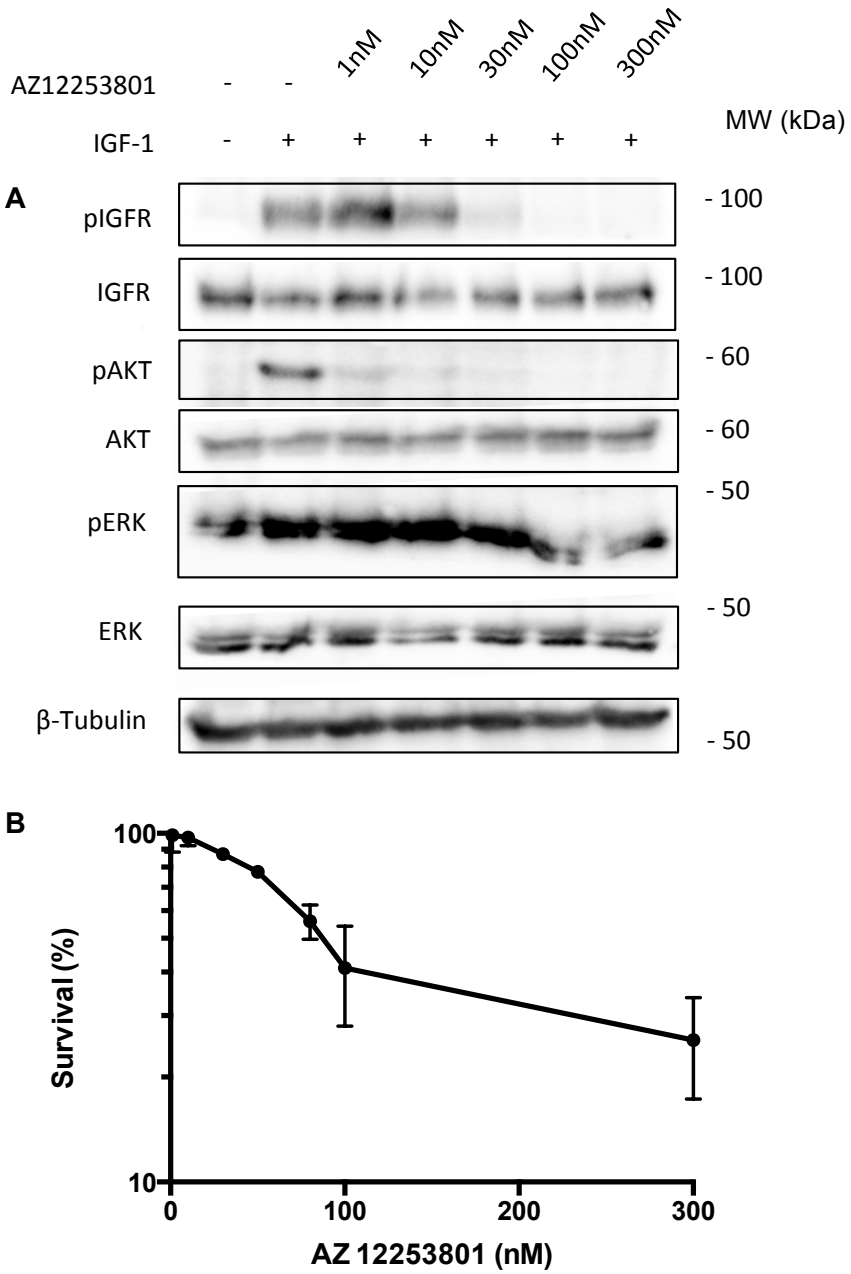


Figure 14: AZ12253801 dose response in SAS cells

A) SAS cells were grown in serum free medium for 24 hours. AZ12253801 was applied, cells were incubated for 45 minutes, then medium was supplemented with 50nM IGF-1 ligand. After 15 minutes, cells were lysed in 3X sample buffer for Western Blotting. Similar results were obtained in a second set of independent lysates. B) SAS cells were seeded at a density of $1.5x10^3$ per $25cm^2$ flask for clonogenic survival assay and after 24 hours treated with AZ12253801. Cells were fixed when control colonies reached ≥50 cells. Results represent the mean ± SEM of triplicate data points for 3 independent assays.

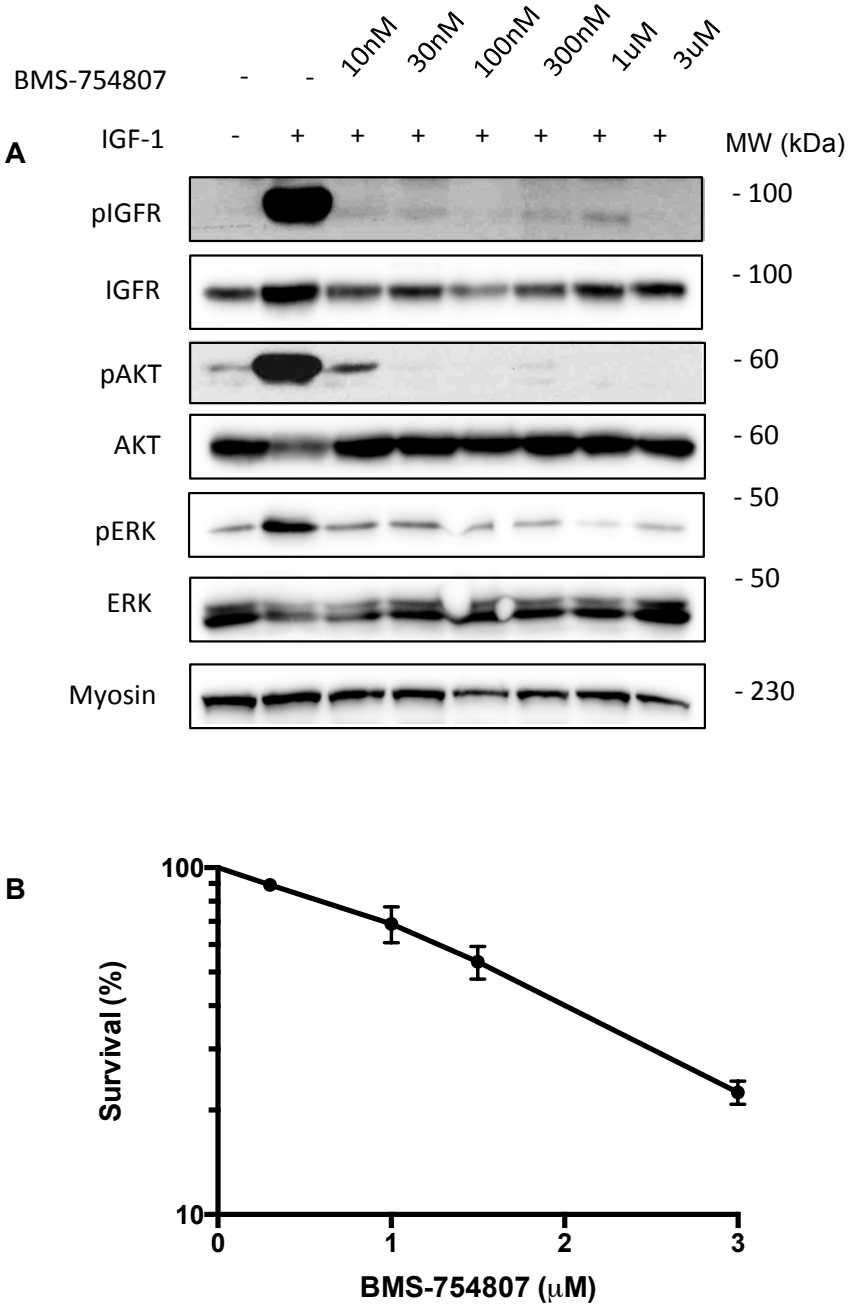


Figure 15: BMS-754807 dose response in SAS cells

A) SAS cells were grown in serum free medium for 24 hours. BMS-754807 was applied, cells were incubated for 45 minutes, then medium was supplemented with 50nM IGF-1 ligand for 15 minutes. Cells were lysed in 3X sample buffer for western blotting. Similar results were obtained in a second set of independent lysates. B) SAS cells were seeded at a density of $1.5x10^3$ in $25cm^2$ flasks for clonogenic survival assay and after 24 hours treated with BMS-754807. Cells were fixed when control colonies reached ≥50 cells. Results represent the mean ± SEM of triplicate data points for 3 independent assays.

The results shown in Figure 15 confirm that BMS-754807 causes target inhibition of IGF-1R and results in a reduction of downstream pAKT and to a lesser extent pERK signalling. BMS-754807 has also been used to inhibit IGF-1R in clinical trials (Haluska et al., 2009), and it was therefore selected for use in further experiments.

A previous study demonstrated variation in the anti-proliferative effect of BMS-754807 between different cancer cell lines (Carboni et al., 2009). In this study, clonogenic survival assay was used to determine the effect of BMS-754807 on HNSCC cell survival. Cells were seeded at the densities described in Table 3, and treated with BMS-754807 at concentrations between 10nM and 3μM. Initial assays in cell line UM-SCC-99 showed that BMS-754807 induced the appearance of large abnormal looking cells that could have been senescent or polyploid. These were so large as to be mis-counted as colonies by the automated colony counter, and therefore this cell line was not used in further assays.

The sensitivity of the remaining six HNSCC cell lines to BMS-754807 varied considerably, with a 40-fold difference between the sensitivity of the most resistant cell line (SAS SF_{50} = 1.59 μM) and the most sensitive cell line (UT-SCC-60A SF_{50} = 40nM) (Figure 16 & Table 22). Evidence from clinical studies suggests that this variation in sensitivity to IGF-1R inhibition may be mirrored clinically (King et al., 2014). Resistance to IGF-1R antibody monotherapy has been identified in patients with advanced HNSCC (Schmitz et al., 2012), despite promising preclinical trials (Riesterer et al., 2011a). These data highlight the need for biomarkers that predict sensitivity to IGF-1R inhibition.

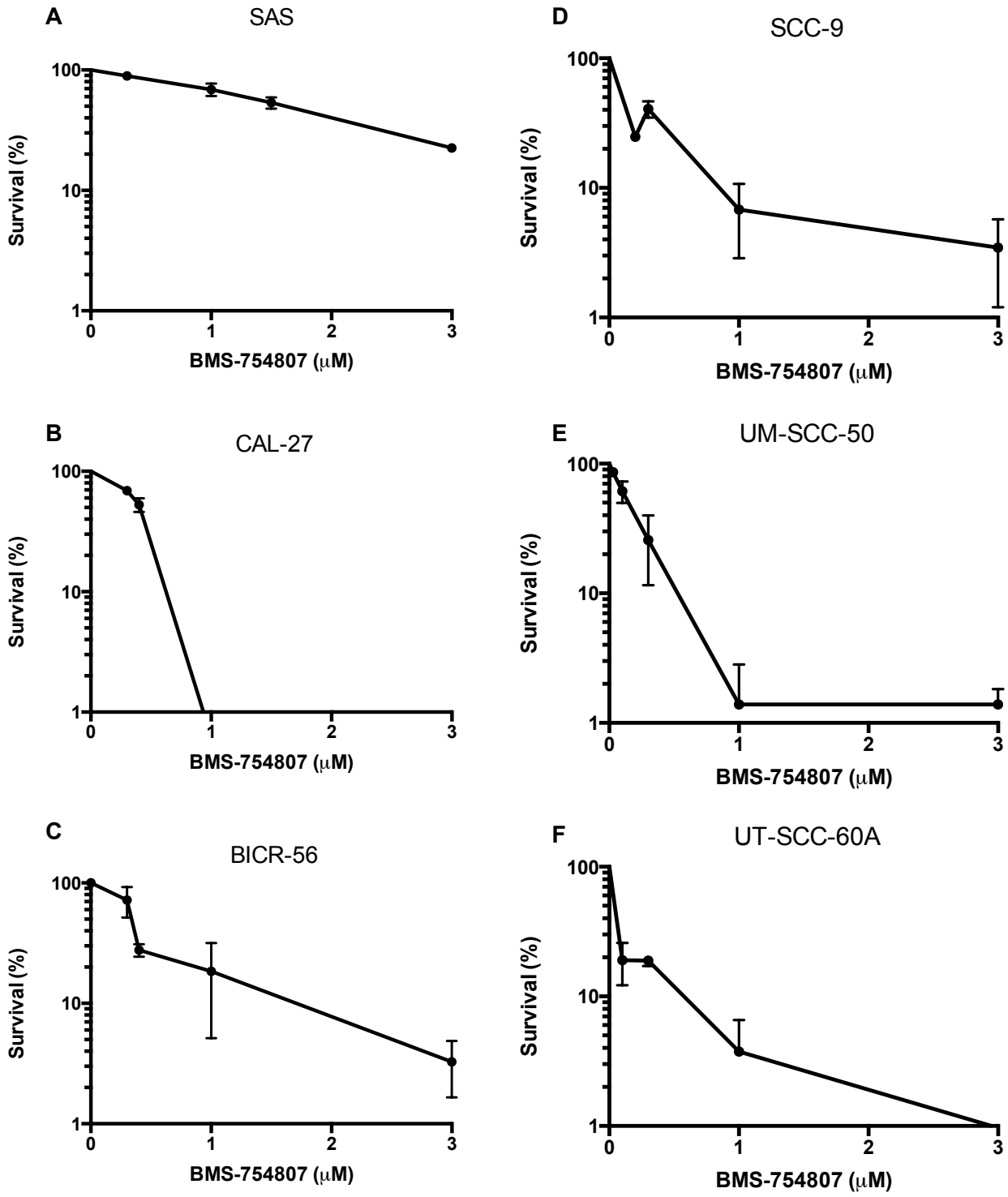


Figure 16: The effect of BMS-754807 on cell survival in HNSCC cell line panel

The HNSCC cell lines SAS (A), CAL-27 (B), BICR-56 (C), SCC-9 (D), UM-SCC-50 (E) and UT-SCC-60A (F) were seeded for clonogenic survival assay in 25cm^2 flasks and treated with BMS-754807. Cells were fixed when colonies reached ≥50 cells in control flasks, treated with solvent alone. Points represent mean ± SEM of pooled data from 2-3 independent assays performed in triplicate.

Cell Line	BMS-754807 SF50 (μM) (95% CI)	AZ12253801 SF50 (μM) (95% CI)
SAS	1.59 (1.41-1.78)	0.08 (0.07-0.09)
CAL-27	0.41 (0.38-0.45)	0.06 (0.05-0.07)
BICR-56	0.35 (0.28-0.45)	Not tested
SCC-9	0.17 (0.14-0.21)	Not tested
UM-SCC-50	0.15 (0.12-0.18)	Not tested
UT-SCC-60A	0.04 (0.03-0.05)	Not tested

Table 22: Sensitivity of HNSCC cell lines to BMS-754807 and AZ12253801

HNSCC cell lines were seeded for clonogenic assay in 25cm flasks and treated with BMS-754807 or AZ12253801. SF_{50} values were calculated as the concentration at which colony counts were 50% of those in solvent treated control flasks. Results show mean values with 95% confidence intervals (95% CI) of 2-3 independent assays performed in triplicate.

5.4 Testing for correlates of sensitivity to BMS-754807 in HNSCC cells

Both translational and clinical studies have demonstrated a significant variation in response to IGF-1R inhibition in cancer cells and clinical tumours, but resistance mechanisms are not understood (Gualberto et al., 2010, Raju et al., 2014). As such, identification of biomarkers indicating the efficacy of IGF-1R inhibition in-vitro and in-vivo is a high priority.

Having defined the protein expression and treatment response of HNSCC cell lines, a correlation analysis was performed to identify biomarkers of response to IGF-1R inhibition. Protein expression on western blotting was quantified using image densitometry (Image J v. 1.46, NIH, USA). For each protein, mean expression from at least two western blots was calculated. Correlation analysis was performed using Pearson correlation coefficient analysis and the results are shown in Table 23. Following Pearson correlation analysis, no statistically significant ($p<0.05$) associations were identified between expression or activation of IGF axis components and sensitivity of HNSCC cell lines to IGF-1R inhibition with BMS-754807. The analysis did, however highlight some non-significant trends. High levels of phosphorylated AKT showed a non-statistically significant association with resistance to IGF-1R inhibition with BMS-754807, with a large effect size ($r= 0.78$, 95% CI -0.078 to 0.98, $p=0.07$). A similar non-significant association was seen with high ERK phosphorylation and resistance to BMS-754807 ($r=0.73$, 95% CI -0.21 to 0.97, $p=0.10$). If it had been possible to access a larger panel of cell lines, it is conceivable that these borderline correlations may have become significant.

	pIGFR	pEGFR	pERK	pAKT	IGFR	EGFR	ERK	AKT	IRS-1	IR	p53
Pearson r	-0.29	0.20	0.73	0.78	0.25	0.14	-0.48	0.28	-0.13	0.37	-0.22
95% CI	-0.89 to 0.68	-0.73 to 0.87	-0.21 to 0.97	-0.078 to 0.98	-0.70 to 0.88	-0.76 to 0.86	-0.93 to 0.55	-0.69 to 0.89	-0.85 to 0.76	-0.63 to 0.91	-0.88 to 0.78
R square	0.08	0.04	0.53	0.61	0.06	0.02	0.23	0.08	0.02	0.14	0.05
P value	0.58	0.70	0.10	0.07	0.63	0.79	0.34	0.59	0.81	0.47	0.67

Table 23: Pearson correlation analysis testing for associations between protein expression and sensitivity to the IGF-1R inhibitor BMS-754807 in HNSCC cell line panel.

Results from this correlation analysis suggest that sensitivity to BMS-754807 is not related to IGF-1R expression or activity in HNSCC cells (r=0.25, 95% CI -0.70 to 0.88, p=0.63 and r=-0.29, 95% CI -0.89 to 0.68, p=0.58 respectively). This supports previously published data suggesting that sensitivity to the IGF-1R monoclonal antibody A12 (Imclone) is not related to IGF-1R expression in HNSCC cell lines (Allen et al., 2007). Other studies have however indicated that IGF-1R expression may be a useful biomarker of sensitivity to IGF-1R inhibition in different tumour types including NSCLC (Gong et al., 2009), breast and colorectal cancer (Zha et al., 2009). Although IGF-1R expression appears to be a useful biomarker for IGF-1R inhibition in some settings, results from this and other studies indicate that IGF-1R is not likely to be a useful predictive biomarker for IGF-1R inhibitor treatment response in HNSCC (Allen et al., 2007).

The trend towards an association between BMS-754807 resistance and AKT phosphorylation is consistent with the findings of Axelrod et al, who performed a targeted screen of 120 drugs in HNSCC cell lines. They found that persistent AKT phosphorylation was associated with reduced response to BMS-754807 in combination with the HER inhibitor BMS-599626 or the Src kinase inhibitor dasatinib (Axelrod et al., 2014). The authors suggest that AKT may represent a 'node of convergence', for IGF-1R and other cell signalling pathways, which could explain de-novo or acquired resistance to IGF-1R inhibition in this setting. Persistent AKT activation by growth factor receptor cross-talk has also been identified as a mechanism of resistance to IGF-1R inhibition by Huang et al (Huang et al., 2009). They demonstrated high levels of AKT phosphorylation in resistant Rh36 (rhabdomyosarcoma) cells treated with the IGF-1R tyrosine kinase inhibitor BMS-536924. The authors suggest that the resistance to IGF-1R inhibition in this cell line relates to EGFR signalling and activation of AKT and MAPK pathways. Clayburgh et al. provide further in-vitro evidence to support this theory in cutaneous HNSCC. They

demonstrated an additive reduction in AKT activation in cell lines treated with the EGFR inhibitor erlotinib and the IGF-1R inhibitor picropodophyllin, and a synergistic reduction in cellular proliferation (Clayburgh et al., 2013). Finally, data from a phase II clinical trial with figitumumab underlines the significance of AKT signalling and IGF-1R resistance in HNSCC patients. In this trial, figitumumab showed no significant clinical activity, but treatment was associated with upregulation of AKT and EGFR activity (Schmitz et al., 2012).

5.5 Testing the effect of IGF-1R inhibition on radiosensitivity of HNSCC cell lines

Up-regulation of IGF-1R is associated with resistance to irradiation in-vitro, and higher risk of local recurrence following lumpectomy and radiotherapy in breast cancer (Turner et al., 1997a). Further evidence for the role of IGF-1R in the radiation response comes from the up-regulation of IGF-1R in non-small cell lung cancer (NSCLC) treated with ionising radiation (Cosaceanu et al., 2007). This is thought to exert a cytoprotective effect through activation of p38 kinase, which promotes DNA damage repair through Ku70/Ku86 DNA binding.

Despite the limited efficacy of IGF-1R inhibition as monotherapy in unselected patients with HNSCC, recent evidence suggests that IGF-1R inhibition may have a role as a radiosensitising agent (Schmitz et al., 2012, Chitnis et al., 2013). Previous work from this laboratory has demonstrated that IGF-1R inhibition or knockdown increases radiosensitivity *in-vitro* and *in-vivo* (Macaulay et al., 2001). IGF-1R inhibition with AZ12253801 in prostate cancer cells led to enhanced radiosensitivity and impaired DNA double strand break repair, characterised by persistent γH2AX foci 24 hours after irradiation (Chitnis et al., 2013). In mouse melanoma cells, down-

regulation of IGF-1R using anti-sense RNA caused increased sensitivity to ionising radiation through reduced activation of ATM kinase (Macaulay et al., 2001).

Before testing the effect of IGF-1R inhibition on radiosensitivity in HNSCC cell lines, the intrinsic radioresistance of each cell line was tested using clonogenic survival assay, which has been identified as the assay of choice for determination of cell survival in radiobiological studies (Franken et al., 2006). It was clear that there was considerable variation in the radiosensitivity of HNSCC cell lines. SAS cells were the most radioresistant of the cell lines tested (SF_{50} DXT = 6.77Gy) and UM-SCC-50 cells were the most radiosensitive (SF_{50} DXT = 1.91Gy) (Table 24).

Cell line	**Dose of irradiation required to achieve 50% cell survival (SF_{50}) (Gy)**
SAS	6.77
CAL-27	3.22
BICR-56	6.68
SCC-9	3.91
UM-SCC-50	1.91
UT-SCC-60A	3.81

Table 24: Sensitivity of HNSCC cells to ionising radiation

In the next set of assays, cell lines were treated with AZ12253801 or BMS-754807, at the pre-determined SF_{50} (Table 22) or DMSO control, and radiosensitisation expressed as a dose-modifying factor (DMF). Multiple t-tests showed significant radiosensitisation of SAS cells with AZ12253801 and BMS-754807. (Figure 17 & Figure 18). Treatment with BMS-754807 reduced the dose of radiotherapy required to achieve 50% cell survival (SF_{50} DXT) from 6.8Gy to 3Gy (DMF = 2.29) in SAS cells, while AZ12253801 reduced the SF_{50} DXT from 5.49Gy to 3.38Gy (DMF = 1.62).

In CAL-27 cells, BMS-754807 also caused significant radiosensitisation, reducing the SF_{50} DXT from 3.2Gy to 2.2Gy (DMF = 1.44) (Figure 18). By contrast, AZ12253801 did not significantly radiosensitise CAL-27 cells (Figure 17), but changed the SF_{50} DXT from 2.99Gy to 2.34Gy (DMF = 1.28) (Table 25).

These results indicate that SAS cells were more effectively radiosensitised than CAL-27 cells. Furthermore the radiosensitising effect of the different IGF-1R tyrosine kinase inhibitors varied; in SAS and CAL-27 cells BMS-754807 resulted in more potent radiosensitisation than AZ12253801 (Table 25). The radiosensitising effect of IGF-1R inhibition was therefore tested in the remaining cell lines using BMS-754807 (Figure 18). Significant radiosensitisation was seen in all cell lines tested, although the magnitude of radiosensitisation varied (Table 25).

The radiosensitising effect of IGF-1R inhibition in HNSCC cell lines has previously been reported (Riesterer et al., 2011b). The authors used an IGF-1R monoclonal antibody (A12, Imclone Systems Inc, NY) in FaDu and HN5 cell lines, and showed a variation in the degree of radiosensitisation achieved. Pre-treatment of cells with 100nM A12 caused significant radiosensitisation in HN5, but not FaDu cells. The variable radiosensitsation achieved with IGF-1R inhibition in HNSCC has also been demonstrated by Raju et al. both *in-vitro* using HN-5, FaDu and Detroit-562 cell lines and mouse xenograft models (Raju et al., 2014). In order to be able to develop this as a clinical approach, it will be important to identify biomarkers to predict the extent to which HNSCC can be radiosensitised by IGF-1R inhibition.

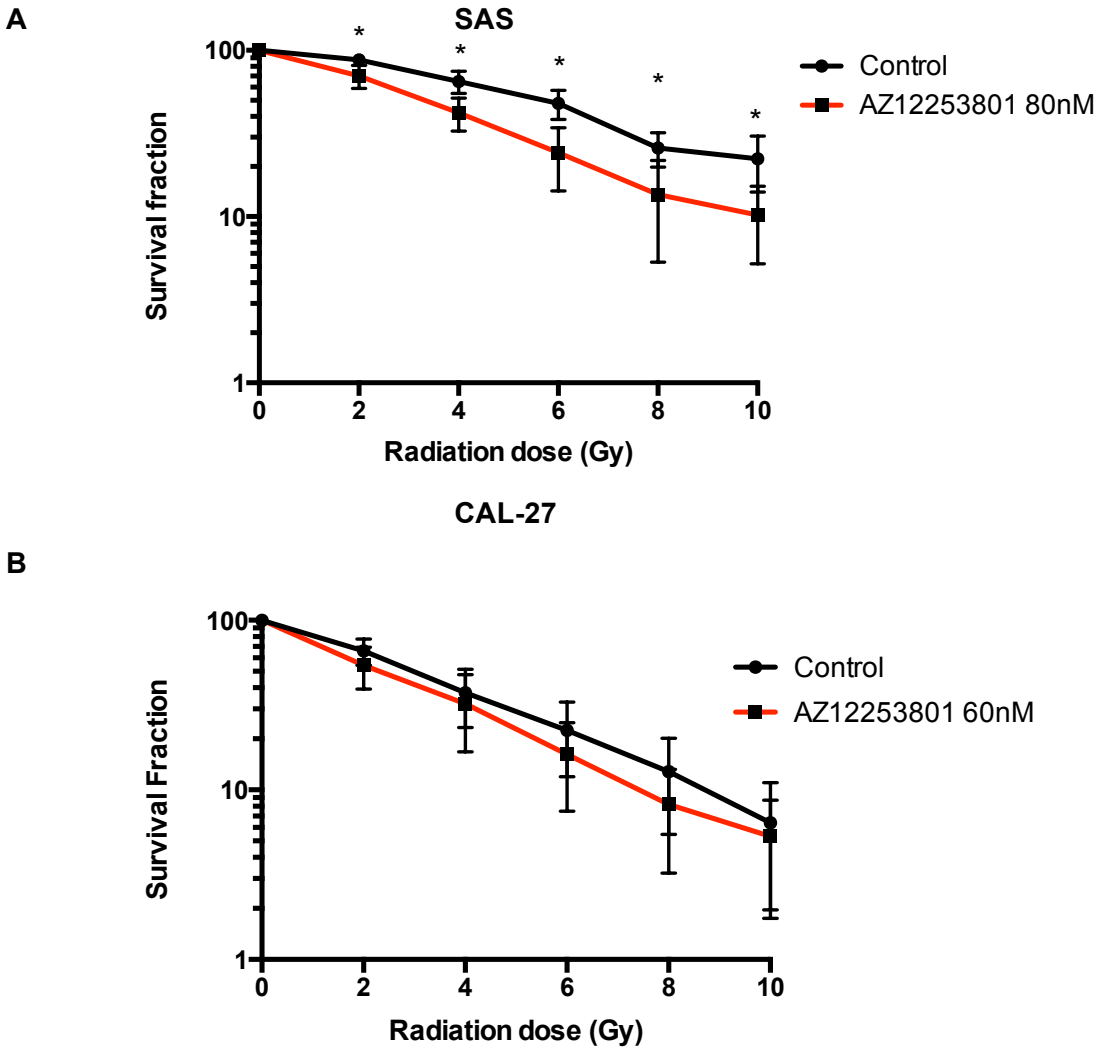


Figure 17: IGF-1R inhibition with AZ12253801 causes radiosensitisation in SAS but not CAL-27 HNSCC cell lines

HNSCC cell lines SAS (A) and CAL-27 (B) were seeded in clonogenic survival assay and treated 24 hours later with AZ12253801 at SF_{50} concentration or DMSO control. After 4 hours incubation, cells were irradiated in a sealed source Caesium-137 irradiator, and fixed when colonies in control flasks reached 50 cells. Survival was expressed as a percentage of the control flasks for each condition. Points represent the mean ± SEM of three assays, each with triplicate data points. Multiple t-tests were used to determine the difference between control and treated cells (*$p<0.05$).

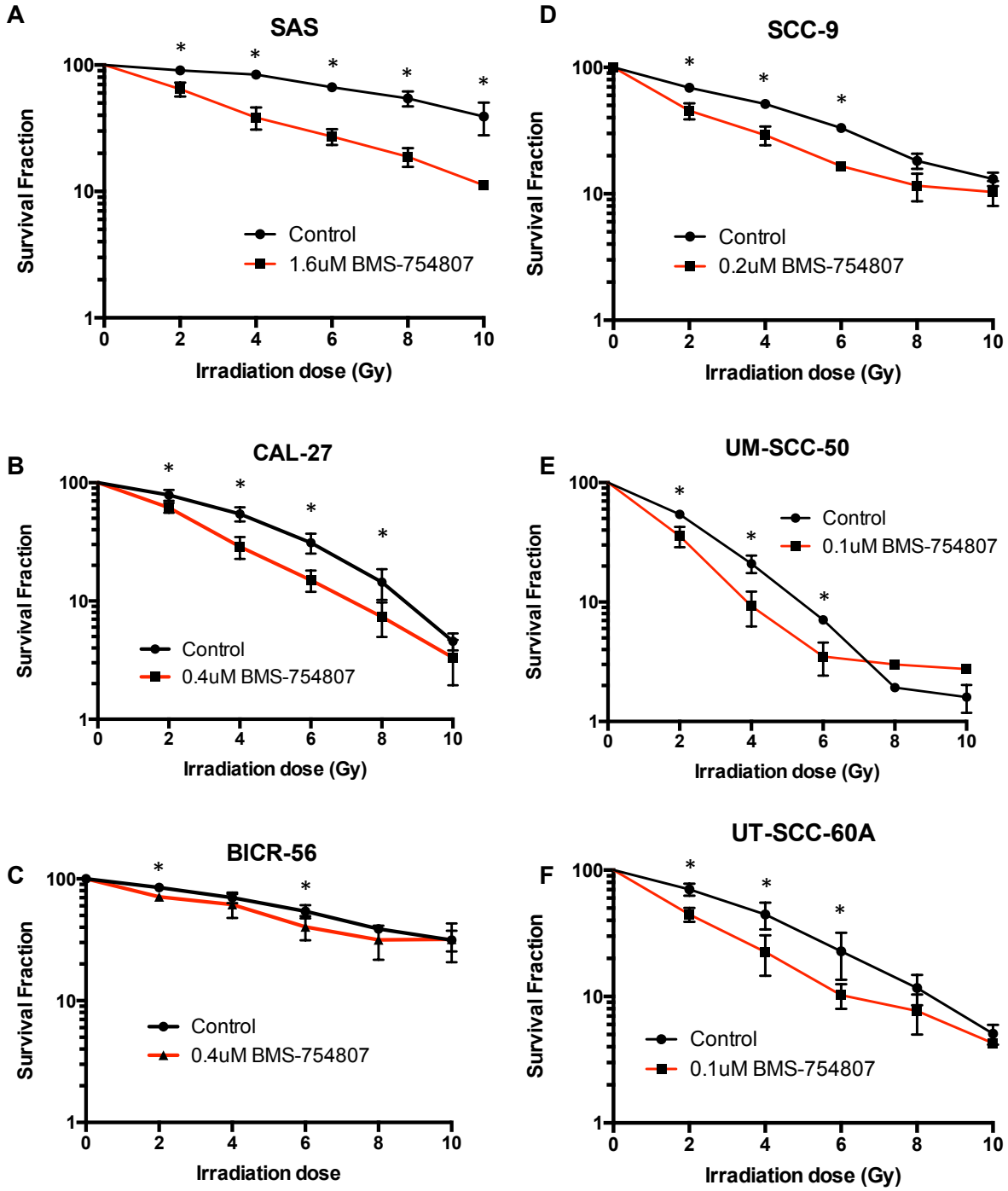


Figure 18: IGF-R inhibition with BMS-754807 causes variable radiosensitisation in HNSCC cell lines.

SAS (A), CAL-27 (B), BICR-56 (C), SCC-9 (D), UM-SCC-50 (E) and UT-SCC-60A (F) cells were seeded in clonogenic survival assay and treated at 24 hours with SF50 BMS-754807 (Table 22) or DMSO control. After 4 hours incubation, cells were irradiated in a sealed source Caesium-137 irradiator, and fixed when colonies in control flasks reached 50 cells. Survival was expressed as a percentage of the control flasks for each condition. Points represent mean ± SEM of three assays each with triplicate data points. Multiple t-tests were used to determine the difference between control and treated cells (*p<0.05).

Cell line	BMS-754807 DMF	AZ12253801 DMF
SAS	2.29	1.62
CAL-27	1.44	1.28
BICR56	1.38	N/A
SCC9	2.09	N/A
UMSCC50	1.59	N/A
UTSCC60A	2.20	N/A

Table 25: Dose modifying factor of the IGF-1R inhibitors BMS-754807 and AZ12253801 on the radiosensitivity of HNSCC cells

5.6 Testing for correlation between IGF axis components and sensitivity of HNSCC cells to irradiation

Pearson correlation analysis of radiosensitivity and protein expression in HNSCC cell lines, suggested a significant association between high ERK phosphorylation and resistance to irradiation (r=0.86, 95% CI: 0.16 to 0.98, p=0.03) (Table 26). A similar but non-significant trend was seen with AKT phosphorylation (r=0.77, 95% CI: -0.11 to 0.97, p=0.07). Increases in both ERK expression and ERK activation have previously been reported in response to ionising radiation (Abbott and Holt, 1999). ERK upregulation leads to activation of downstream effector proteins including P90RSK, which in turn activates anti-apoptotic proteins (Riccio et al., 1999). This is thought to exert a cytoprotective effect, in squamous cell carcinoma following irradiation (Park et al., 1999). Phosphorylation of AKT may also play a role in radioresistance in HNSCC. Recent evidence suggests that high levels of AKT phosphorylation in tumour tissue from patients with HNSCC treated with radiotherapy are associated with reduced overall survival and progression free survival (Freudlsperger et al., 2014). These data indicate that activation of pro-survival pathways downstream of IGF-1R are associated with radioresistance in HNSCC. In the current study, however, there was no association between IGF-1R expression or phosphorylation and radiosensitivity in HNSCC cell lines. This finding is consistent with the IGF-1R immunohistochemistry results from Chapter 4, which indicate that IGF-1R expression is not independently associated with adverse survival in HNSCC patients. In order to more specifically test associations with radioresistance, it would be necessary to compare outcomes post-irradiation in patients with high vs low IGF-1R tumours; or conversely to assess IGF-1R immunohistochemistry in patients experiencing early relapse vs desirable disease control post-irradiation.

	pIGFR	pEGFR	pERK	pAKT	IGFR	EGFR	ERK	AKT	IRS-1	IR	p53
Pearson r	0.23	-0.03	0.86	0.77	-0.04	-0.10	-0.35	0.13	-0.38	0.42	-0.62
95% CI	-0.72 to 0.88	-0.82 to 0.80	0.16 to 0.98	-0.11 to 0.97	-0.83 to 0.80	-0.84 to 0.78	-0.90 to 0.65	-0.76 to 0.85	-0.91 to 0.62	-0.59 to 0.92	-0.95 to 0.39
R square	0.05	0.00	0.74	0.59	0.00	0.01	0.12	0.02	0.15	0.18	0.38
P value	0.66	0.96	0.03	0.07	0.93	0.85	0.50	0.81	0.46	0.41	0.19

Table 26: Pearson correlation analysis testing for associations between protein expression and radioresistance in HNSCC cell line panel.

5.7 Testing for correlation between IGF axis components and sensitisation of HNSCC cells to radiotherapy by BMS-754807

The results of the correlation analysis incorporating the dose-modifying factor of BMS-754807 on radiosensitivity are shown in Table 27. This showed no statistically significant associations between protein expression in the HNSCC cell lines and the degree of radiosensitisation achieved with BMS-754807. Similar to the previous analysis, however, the expression of some proteins suggested a non-statistically significant trend. The association between IGF-1R expression and the dose-modifying effect of BMS-754807 did not reach statistical significance (r=-0.76, 95%CI -0.97 to 0.13, p=0.08), but suggested a possible association between high IGF-1R expression and reduced radiosensitisation upon IGF-1R inhibition. Analysis of the relationship between IGF-1R activity (pIGF-1R) and BMS-754807 DMF however, showed no association, indicating that the trend towards an association with IGF-1R expression should be interpreted with caution. Pearson correlation coefficient analysis demonstrated no association between radiosensitivity of HNSCC cell lines and the dose-modifying factor of BMS-754807 (r = 0.16, 95% CI: -0.77 to 0.86, p = 0.75), suggesting that baseline radiosensitivity does not affect the degree to which HNSCC cell lines are radiosensitised by IGF-1R inhibition.

Analysis of EGFR expression and activity also showed a possible link with radiosensitisation, although this too failed to reach statistical significance. A trend towards reduced radiosensitisation by BMS-754807 was seen in cell lines expressing high levels of EGFR and pEGFR (EGFR: r=-0.74, 95%CI -.097 to 0.18, p=0.09, pEGFR: r=-0.67, 95%CI -.096 to 0.31, p=0.14). Although not statistically significant,

these results would fit with those of previous studies which suggest that EGFR signalling in HNSCC may provide an 'escape' from sensitivity to IGF-1R inhibition (Schmitz et al., 2012). In the context of irradiation, these findings could be explained by the effect of EGFR signalling on DNA double strand break repair: In response to DNA damage, EGFR translocates to the nucleus and interacts with DNA-dependent protein kinase (DNA-PK) to promote non-homologous end-joining (NHEJ) (Mukherjee et al., 2010). Consistent with this effect, EGFR inhibition with cetuximab, causes delayed DSB repair in irradiated HNSCC cells, with persistence of γH2AX and 53BP1 foci at 24 hours (Raju et al., 2014). These data highlight the role of the EGFR pathway in cell survival following irradiation, and suggest a possible mechanism by which HNSCC cells could overcome IGF-1R inhibitor induced radio-sensitisation.

	pIGFR	pEGFR	pERK	pAKT	IGFR	EGFR	ERK	AKT	IRS-1	IR	p53
Pearson r	0.04	-0.67	0.11	0.08	-0.76	-0.74	-0.51	-0.03	-0.40	-0.36	-0.75
95% CI	-0.80 to 0.82	-0.96 to 0.31	-0.77 to 0.85	-0.78 to 0.84	-0.97 to 0.13	-0.97 to 0.18	-0.93 to 0.52	-0.82 to 0.80	-0.92 to 0.61	-0.91 to 0.64	-0.97 to 0.16
R square	0.00	0.45	0.01	0.01	0.58	0.55	0.26	0.00	0.16	0.13	0.56
P value	0.94	0.14	0.83	0.87	0.08	0.09	0.30	0.95	0.43	0.49	0.09

Table 27: Pearson correlation analysis testing for associations between protein expression and radiosensitisation induced by the IGF-1R inhibitor BMS-754807 in HNSCC cell line panel.

5.8 Selection of biomarker candidates for further analysis

Results from the correlation analyses suggest possible associations between protein expression and phenotype in HNSCC cell lines. Activation of pro-survival, anti-apoptotic signalling proteins was associated with radioresistance, and a trend towards resistance to IGF-1R inhibition in this cell line panel.

The drivers of AKT and ERK activation in HNSCC may be complex and likely to vary between tumours, depending on the genetic and epigenetic changes in particular tumours (Leemans et al., 2011). In general, activation of signalling proteins may result from membrane-bound receptor signalling or constitutive activation of downstream effector proteins.

Amplification of EGFR resulting in oncogenic activation has been reported in up to 30% of HNSCC (Sheu et al., 2009). The prevalence of activating point mutations of EGFR in HNSCC, however, is thought to be lower, affecting 1% of Caucasians with HNSCC (Loeffler-Ragg et al., 2006). EGFR activation in this way may lead to enhanced signalling down both the PI3K-AKT and RAS-MAPK pathways, resulting in cell survival and proliferation. There is accordingly, an association between increased EGFR expression and adverse survival in HNSCC (Leemans et al., 2011).

Constitutive activation of downstream signalling pathways may also provide a mechanism for resistance to IGF-1R inhibition. MAPK and PI3K signalling drive cellular survival and proliferation and are characterised by ERK and AKT phosphorylation respectively. Activating mutations in oncogenes in these pathways result in deregulation of effector proteins, resulting in the key characteristics of cancer including stimulation-independent proliferation, avoidance of apoptosis, invasion and metastasis (Hanahan and Weinberg, 2000). One such oncogene is

RAS, which has been identified as a critical regulator of cell signaling (Downward, 2003). Deregulation of RAS occurs in the majority of human cancers, and may occur due constitutive upstream activation, or oncogenic RAS mutations. Up to 22% of HNSCC harbor RAS mutations, in the majority of cases in the HRAS isoform (Anderson et al., 1994, Prior et al., 2012). The therapeutic significance of HRAS mutation in HNSCC has recently been studied by Hah et al, who found that HRAS mutation is associated with resistance to EGFR inhibition in HNSCC (Hah et al., 2014).

The results of the correlation analyses described in Sections 5.6 & 5.7 suggest that phosphorylation of ERK and AKT may play a role in and possibly contribute to resistance to IGF-1R inhibition and ionising radiation. It is clear that previously published studies have highlighted the significance of EGFR (Hama et al., 2009) and RAS (Hah et al., 2014) signalling pathways in HNSCC. Both of these signalling pathways are associated with AKT and ERK phosphorylation, and therefore were selected for further evaluation in the context of resistance to IGF-1R inhibition in HNSCC.

5.9 Co-inhibition of IGF-1R and EGFR in HNSCC cell lines

The results from this study correlating protein expression and HNSCC cell phenotype suggest that EGFR expression or activation may be associated with a reduced ability to achieve radiosensitisation with IGF-1R inhibition. In order to test this hypothesis, EGFR was inhibited or depleted using the EGFR tyrosine kinase inhibitor Gefitinib and EGFR short-interfering RNA (siRNA). Therefore the next set of experiments used EGFR siRNA alone or in combination with BMS-754807 in SAS and CAL-27 cells. Two different siRNAs were used: siEGFR_HS10 and siEGFR_HS11. Each achieved partial EGFR depletion in CAL-27 cells (Figure 19).

IGF-1R inhibition with BMS-754807 achieved modest radiosensitisation of CAL-27 cells (DMF=1.25) (Figure 19A), similar to that seen previously (Figure 18B). EGFR depletion with si-EGFR_HS10 did not enhance radiosensitivity (DMF=0.77), nor did it increase the effect of BMS-754807 (DMF=1.26). Similarly, EGFR depletion with siEGFR_HS11 caused no additional radiosensitisation in combination with BMS-754-807 (DMF=2.37). It did, however result in modest radiosensitisation in combination with irradiation (DMF=1.26) (Figure 19B).

In SAS cells, more effective knockdown of EGFR was achieved (Figure 20A & B). BMS-754807 caused radiosensitisation of SAS cells, although the magnitude of this effect was smaller than previously observed (DMF=1.83) (Figure 18A). This may reflect the toxicity of the transfection protocols. Using siEGFR_HS11, no further radiosensitisation was observed with BMS-754807 (DMF=1.58). In order to increase EGFR knockdown, dual (reverse, then forward) transfection was performed with siEGFR_HS10 and siEGFR_HS11. This resulted in improved EGFR depletion, but no radiosensitisation (in the absence of IGF-1R inhibition) (DMF=0.96), and no additional radiosensitisation with BMS-754807 (DMF=1.78).

These experiments suggest that knockdown of EGFR in CAL-27 and SAS cell lines did not increase the degree of radiosensitisation achieved with BMS-754807. However, EGFR depletion was only partial, and therefore a further set of experiments was performed using the EGFR inhibitor Gefitinib, in an attempt to more completely block EGFR signalling.

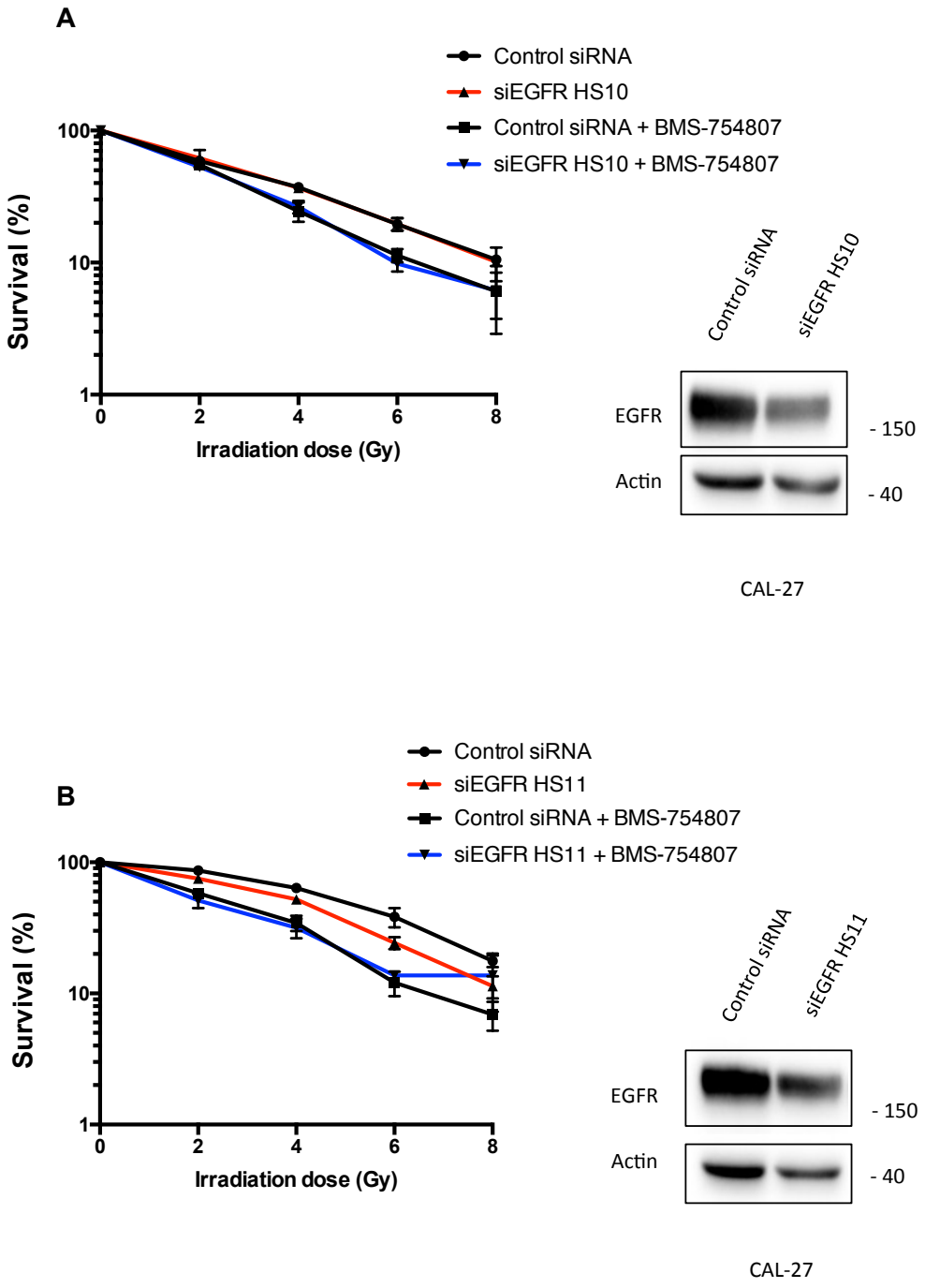


Figure 19: EGFR knockdown in CAL-27 HNSCC cells using short-interfering RNA

CAL-27 cells were transfected with control siRNA and A) siEGFR_HS10, B) siEGFR_HS11. Cells were incubated for 48 hours, disaggregated and seeded into clonogenic survival assay. The remainder were collected for western blotting. After 24 hours, cells were treated with BMS-754807 or solvent control (0.01% DMSO), incubated for 4 hours and irradiated. Results represent the mean ± SEM of a single assay performed in triplicate. DMF values for BMS-754807, EGFR depletion and the combination were A) 1.25, 0.77 & 1.26 and B) 1.98, 1.26 & 2.37 respectively. Multiple t-tests showed no significant difference in the radiosensitisation achieved with BMS-754807 and EGFR knockdown compared to BMS-754807 alone.

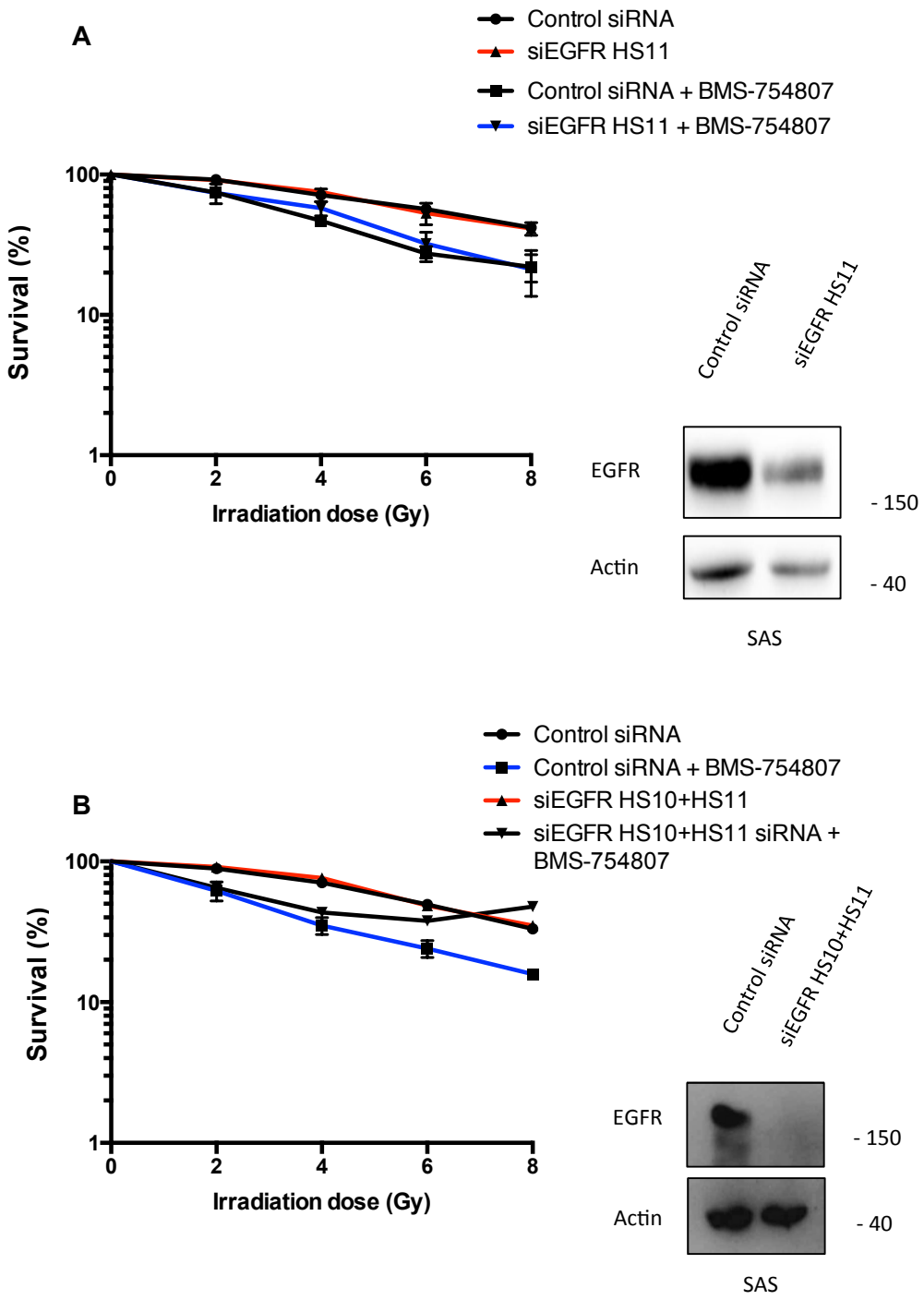


Figure 20: EGFR knockdown in SAS HNSCC cells using short-interfering RNA

SAS cells were transfected with control siRNA and A) siEGFR_HS11, B) siEGFR_HS10 and siEGFR_HS11. Cells were incubated for 48 hours, disaggregated and seeded into clonogenic survival assay. The remainder were collected for western blotting. After 24 hours, cells were treated with BMS-754807 or solvent control (0.01% DMSO), incubated for 4 hours and irradiated. Results represent the mean ± SEM of a single assay performed in triplicate. DMF values for BMS-754807, EGFR depletion and the combination were A) 1.83, 1.00 & 1.58 and B) 2.22, 0.96 & 1.78 respectively. Multiple t-tests showed no significant difference in the radiosensitisation achieved with BMS-754807 and EGFR knockdown compared to BMS-754807 alone.

CAL-27 cells were chosen for this analysis due to the high levels of total EGFR and phosphorylated EGFR observed in this cell line, and the modest radiosensitsation achieved with BMS-754807 (Figure 12 & Figure 17). EGFR inhibition was achieved with Gefitinib, which resulted in a dose-dependent reduction of downstream signalling and cell survival (SF50 = 54nM) (Figure 21 A & B). The combination of 0.4μM BMS-754807 and 50nM Gefitinib in CAL-27 cells caused almost complete inhibition of cell survival in non-irradiated cells (mean cell survival as a percentage of untreated controls: 1.8% ± 0.3) (Figure 22A). In irradiated cells treated with both inhibitors, survival evaluation was not possible due to the low level of cell survival in control flasks. However, assays using each agent separately (Figure 22B) confirmed significant, although modest radiosensitisation in CAL-27 cells treated with BMS-754807 (DMF = 1.75), and limited radiosensitisation upon Gefitinib treatment (DMF = 1.17). Therefore, the experiments were repeated using both inhibitors at SF75 concentrations (the concentration of drug required to reduce the surviving fraction of colonies to 75%) (Figure 23). The combination of BMS-754807 and Gefitinib at SF75 concentrations caused a supra-additive reduction in cell survival in non-irradiated cells; mean cell survival in cells treated with 0.2μM BMS-754807 was 67%, with 20nM Gefitinib it was 71%, and with co-treatment was 18% (Figure 23A). Despite this, co-treatment with both inhibitors did not increase the radiosensitisation seen with BMS-754807. Indeed, at SF75 neither BMS-754807 (DMF=1) nor Gefitinib (DMF=1) increased the sensitivity of CAL-27 cells to radiotherapy (Figure 23B).

The results from the experiments presented in this section suggest that neither the expression nor the activity of EGFR predict the ability to radiosensitise HNSCC cells with BMS-754807. In addition, they indicate that dual inhibition of EGFR and IGF-1R may not be an effective means of increasing the degree of radiosensitisation achieved.

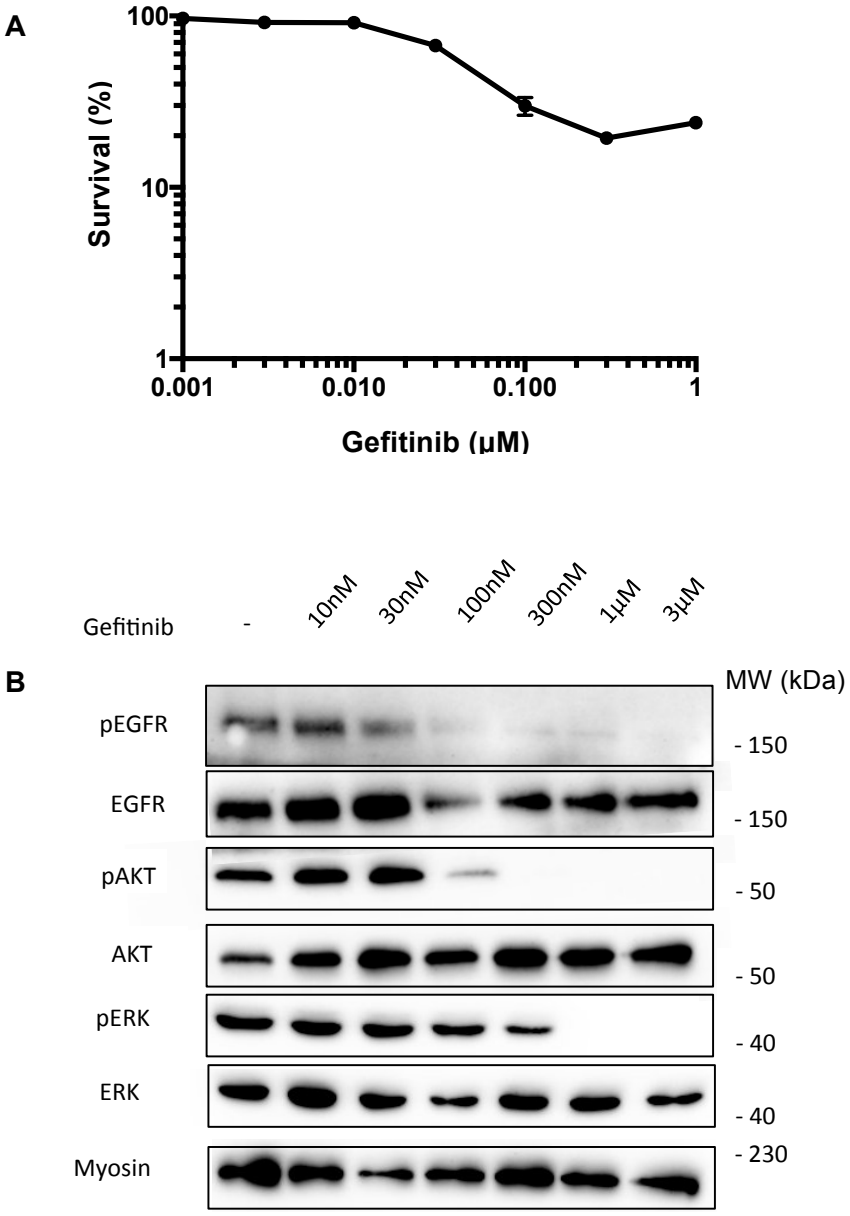


Figure 21: Gefitinib dose response in CAL-27 HNSCC cells.

(A) CAL-27 cells were seeded for clonogenic survival assay and the following day were treated with Gefitinib at concentrations of 1nM-1µM. Results represent the mean ± SEM of 3 assays with triplicate data points. Dose-dependent inhibition of cell survival was observed (SF50 = 54nM, SF75 = 21nM). (B) Western blot showing cell-signalling effects of Gefitinib on CAL-27 cells. Cells were grown in serum-free medium overnight, then treated with Gefitinib for 45 minutes and additionally stimulated with 10nM EGF ligand for 15 minutes. Lysates were analysed by western blotting using myosin as a loading control.

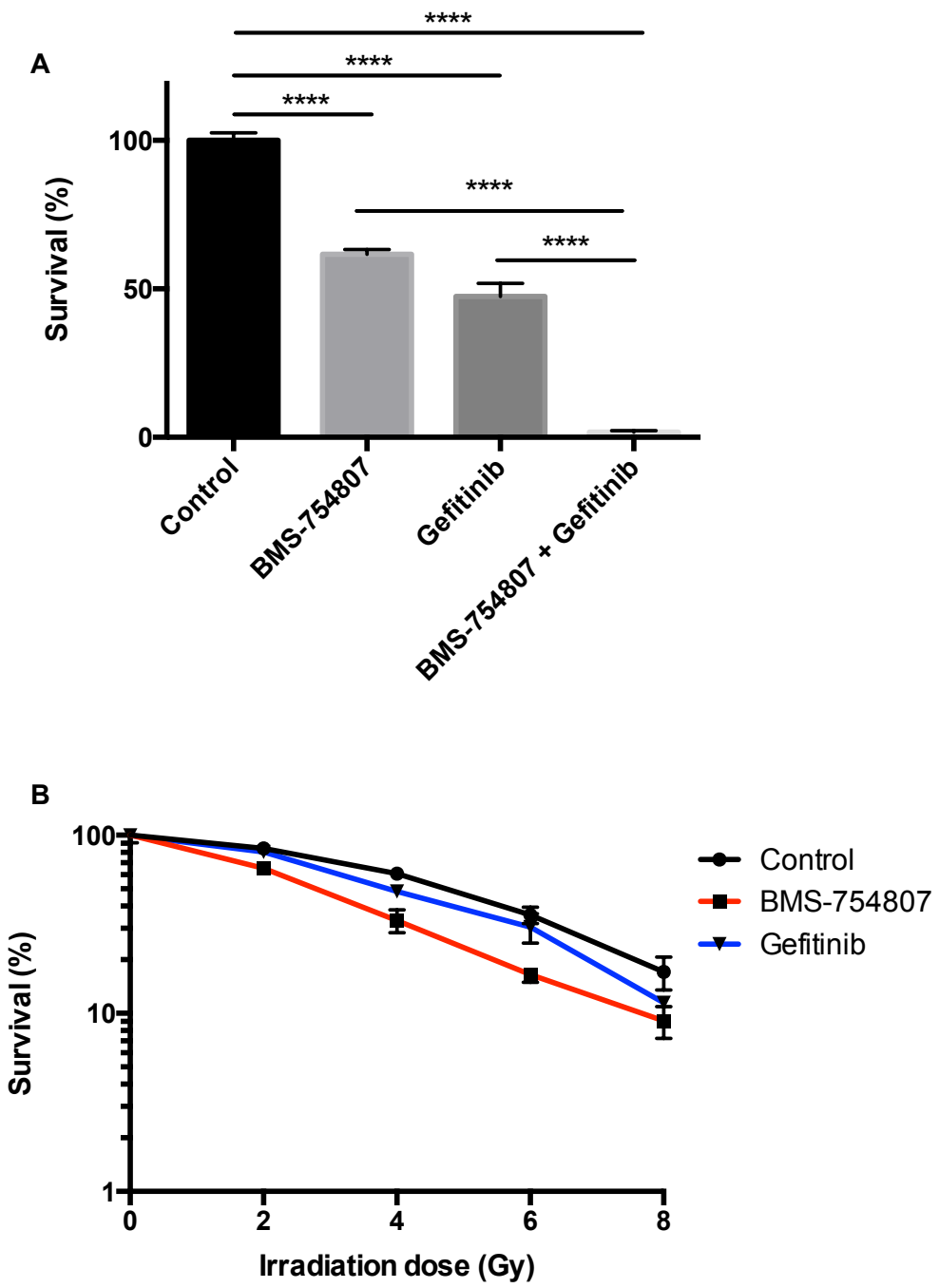


Figure 22: Combination treatment of CAL-27 cells with BMS-754807 and Gefitinib at SF_{50} concentrations

(A) Clonogenic survival assay showing the effect of 0.4μM BMS-574807, 50nM Gefitinib and the combination of both inhibitors on cell survival. At SF_{50} concentrations, combination treatment caused complete inhibition of cell survival. Combination treatment significantly reduced cell survival compared to each agent alone (****$p<0.0001$) (B) The effect of BMS-754807 and Gefitinib on radiosensitivity of CAL-27 cells. At SF_{50} concentrations BMS-754807 caused significant radiosensitisation at 2,4,6 & 8Gy ($p<0.05$) (DMF=1.75). Gefitinib caused significant radiosensitisation at 4Gy ($p<0.05$) but the magnitude of this effect was lower (DMF=1.17). Results are shown from two independent assays performed in triplicate.

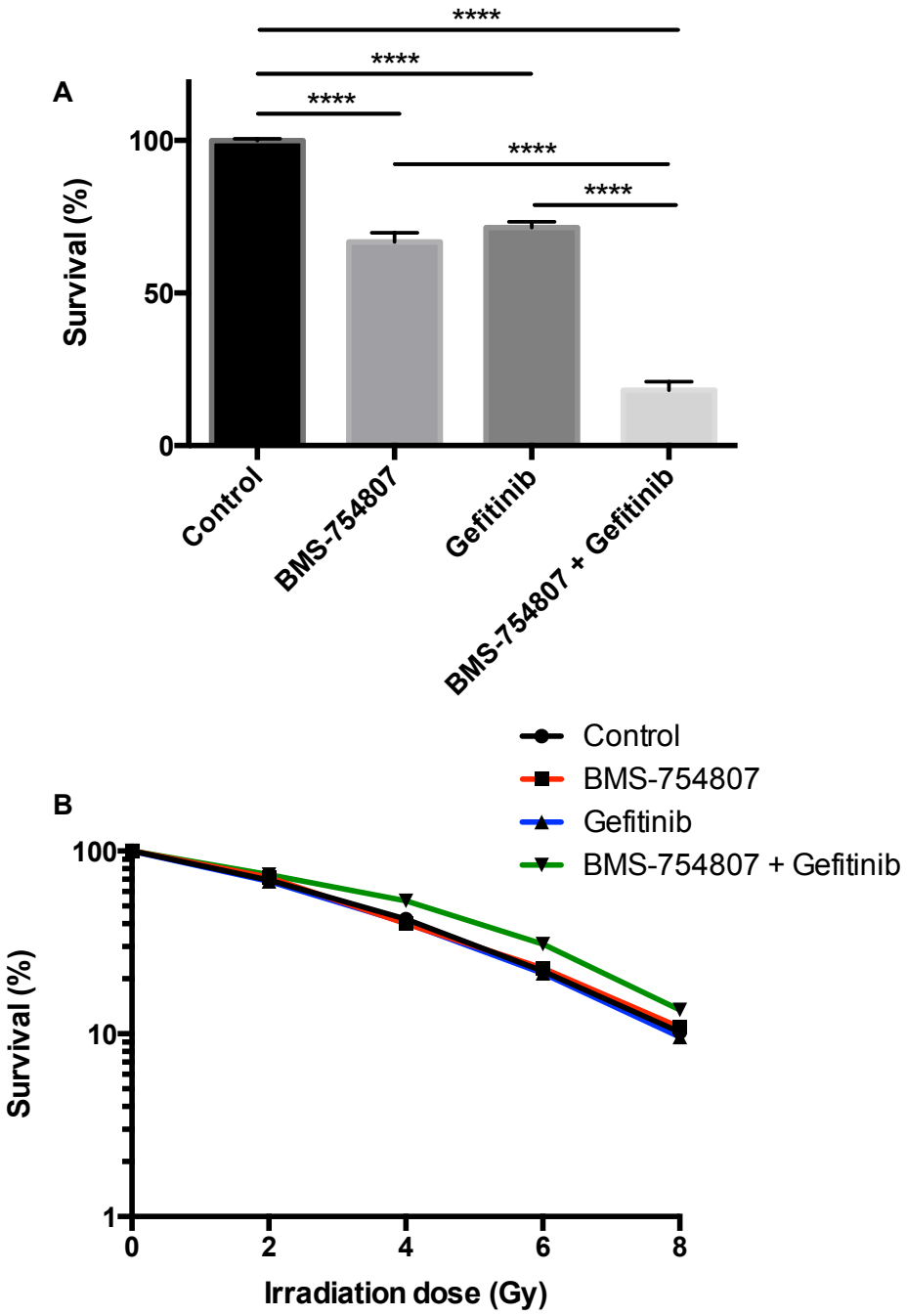


Figure 23: Combination treatment of CAL-27 cells with BMS-754807 and Gefitinib at SF_{75} concentrations

(A) Clonogenic survival assay showing the effect of 0.2μM BMS-574807, 20nM Gefitinib and the combination of both inhibitors on cell survival. At SF_{75} concentrations, combination treatment caused significant inhibition of cell survival. Combination treatment significantly reduced cell survival compared to each agent alone (****p<0.0001) (B) The effect of BMS-754807 and Gefitinib on radiosensitivity of CAL-27 cells. At SF_{75} concentrations neither BMS-754807 (DMF=1) nor Gefitinib (DMF=1) caused significant radiosensitisation. Combination treatment did not enhance radiosensitivity. Results are shown from a single assay performed in triplicate.

5.10 HRAS as a predictive biomarker for sensitivity to IGF-1R inhibition

The results from correlation analysis (Sections 5.4 & 5.6) and data recently published by Hah et al indicate that HRAS may influence sensitivity to IGF-1R inhibition in HNSCC cells (Hah et al., 2014). The next experiment aimed to test the effect of activating HRAS mutations on the sensitivity of HNSCC cells to IGF-1R inhibition using an isogenic model. To select a suitable host cell line, the mutation status of all cell lines in the cell line panel was tested using next generation sequencing, and the results are shown in

Table 28. CAL-27 and SAS cells harboured synonymous *HRAS* mutations neither of which resulted in a change to the encoded amino acid, and CAL-27 also harboured a missense *NRAS* mutation. The remainder of the cell lines (BICR-56, SCC-9, UM-SCC-50 & UT-SCC-60A) had no detectable RAS mutations.

Missense *p53* mutations were detected in CAL-27 and UM-SCC-50 cells, which were the two cell lines that had detectable p53 protein on initial analysis (Figure 12). Although the *p53* mutation status of UM-SCC-50 cells has not previously been reported, a missense *p53* mutation has been demonstrated in CAL-27 cells (Yip et al., 2006). This may lead to production of a full-length, altered p53 protein, with a prolonged half-life, which exerts a tumorigenic effect by interaction with other intracellular proteins, or directly influencing gene transcription. (Freed-Pastor and Prives, 2012).

The SAS and UT-SCC-60A cell lines were chosen to test the effect of HRAS mutation on sensitivity to BMS-754807. Viral vectors encoding HRAS G12D, HRAS G12V, wild type HRAS or empty vector were obtained from Professor Jeffrey N. Myers (MD Anderson Cancer Centre, Texas, US). These were used to make virus as

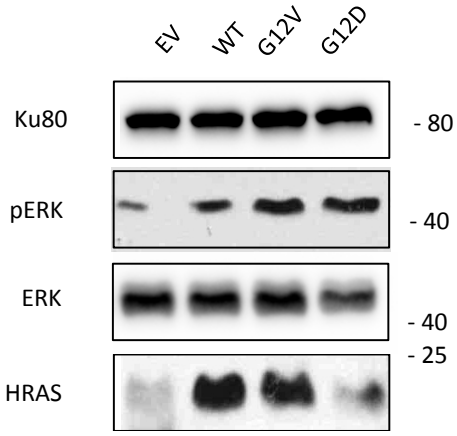
described in Materials and Methods, and viral supernatants were used to infect the HNSCC cells. The results are shown in (Figure 24 & Figure 25).

Sample ID	Chromosome position	Gene	Coding	Amino Acid change	Function
CAL-27	5:34242	HRAS	c.81T>C	H27H	Synonymous
	11:5256508	NRAS	c.203G>C	R68T	Missense
	5:5599284	KIT	c.2410C>T	R804W	Missense
	21:971153	CDKN2A	c.205G>T	E69	Nonsense
	7:578271	TP53	c.578A>T	H193L	Missense
	4:8584560	SMAD4	c.733C>T	Q245	Nonsense
SAS	5:34242	HRAS	c.81T>C	H27H	Synonymous
	7:574021	TP53	c.1006G>T	E336	Nonsense
UM-SCC-	17:7577098	TP53	c.444A>T	R148S	Missense
	17:7578189	TP53	c.381T>A	Y127	Nonsense
UT-SCC-	3:178928080	PIK3CA	c.1358A>C	E453A	Missense
BICR-56	7:116340262	MET	c.1124A>G	N375S	Missense
	9:21970971	CDKN2A	c.387C>A	Y129	Nonsense
SCC-9	3:178927410	PIK3CA	c.1173A>G	I391M	Missense

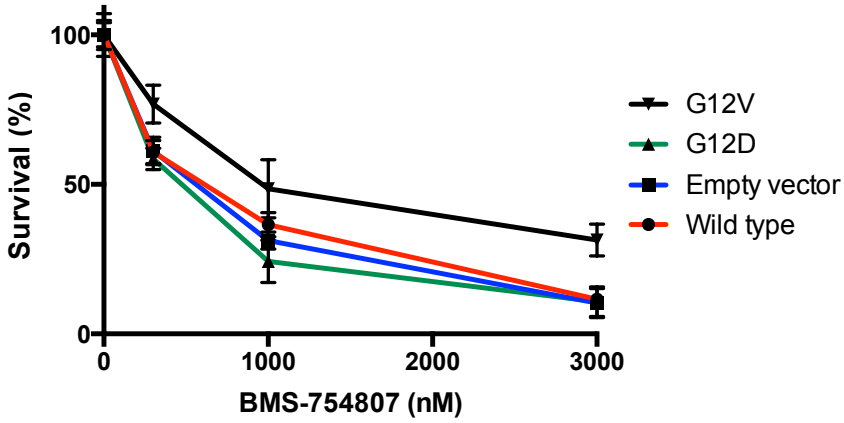
Table 28: Mutation detection in HNSCC cell line panel.

Genomic DNA was extracted from cells as described in Materials and Methods. DNAs were analysed using next generation sequencing for a panel of 46 cancer-associated mutations by Dr Anthony Cutts, Molecular Pathology, John Radcliffe Hospital, Oxford. RAS mutations were identified in two cell lines: SAS cells harboured a synonymous HRAS mutation, which did not result in a change in the encoded amino acid. A similar mutation was seen in CAL-27 cells, which also harboured a missense NRAS mutation.

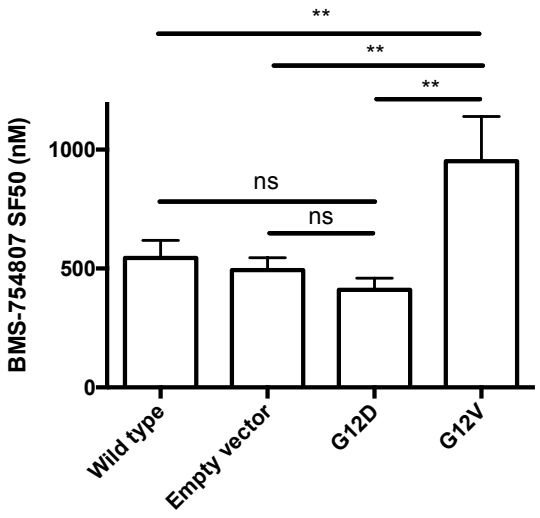
A


EV
WT
G12V
G12D
Ku80
pERK
ERK
HRAS
- 80
- 40
- 40
- 25

B


Survival (%)
100
50
0
0
1000
2000
3000
BMS-754807 (nM)
G12V
G12D
Empty vector
Wild type

C


BMS-754807 SF50 (nM)
1000
500
0
**
**
**
ns
ns
Wild type
Empty vector
G12D
G12V

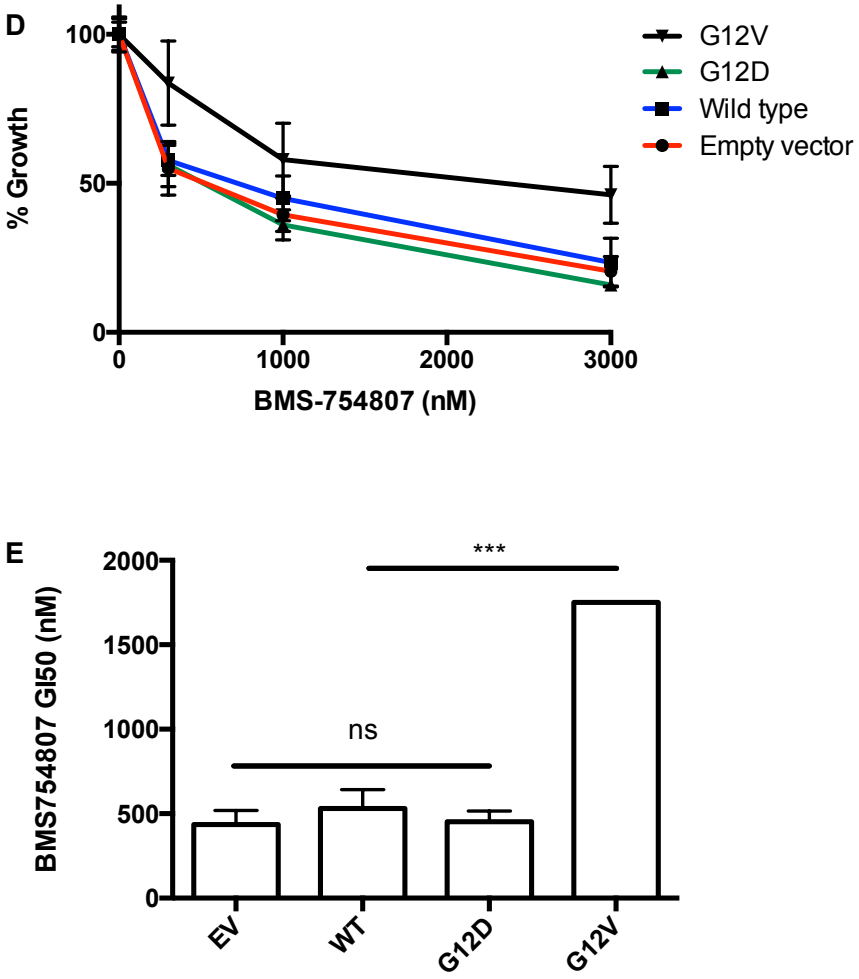


Figure 24: Effect of mutant HRAS expression on response to BMS-754807in SAS cells.

SAS cells were infected with mutant HRAS (G12D or G12V), wild type HRAS or empty vector. (A) Western blot showing expression of HRAS in infected cells and upregulation of pERK in HRAS G12V & G12D. (B) Clonogenic survival assay showing dose response of infected cells to BMS-754807. Points represent mean values ± SEM from 2 independent assays performed in triplicate. (C) SF_{50} values from clonogenic survival assay; cells infected with HRAS G12V were significantly more resistant to BMS-754807 (**p<0.01). (D) CellTiter Glo proliferation assay showing dose response of transfected cells to BMS-754807. Points represent mean values ± SEM from 3 independent assays performed in triplicate. (E) GI_{50} values from CellTiter Glo assay showing resistance of HRAS G12V mutant to BMS-754807 (***p<0.001).

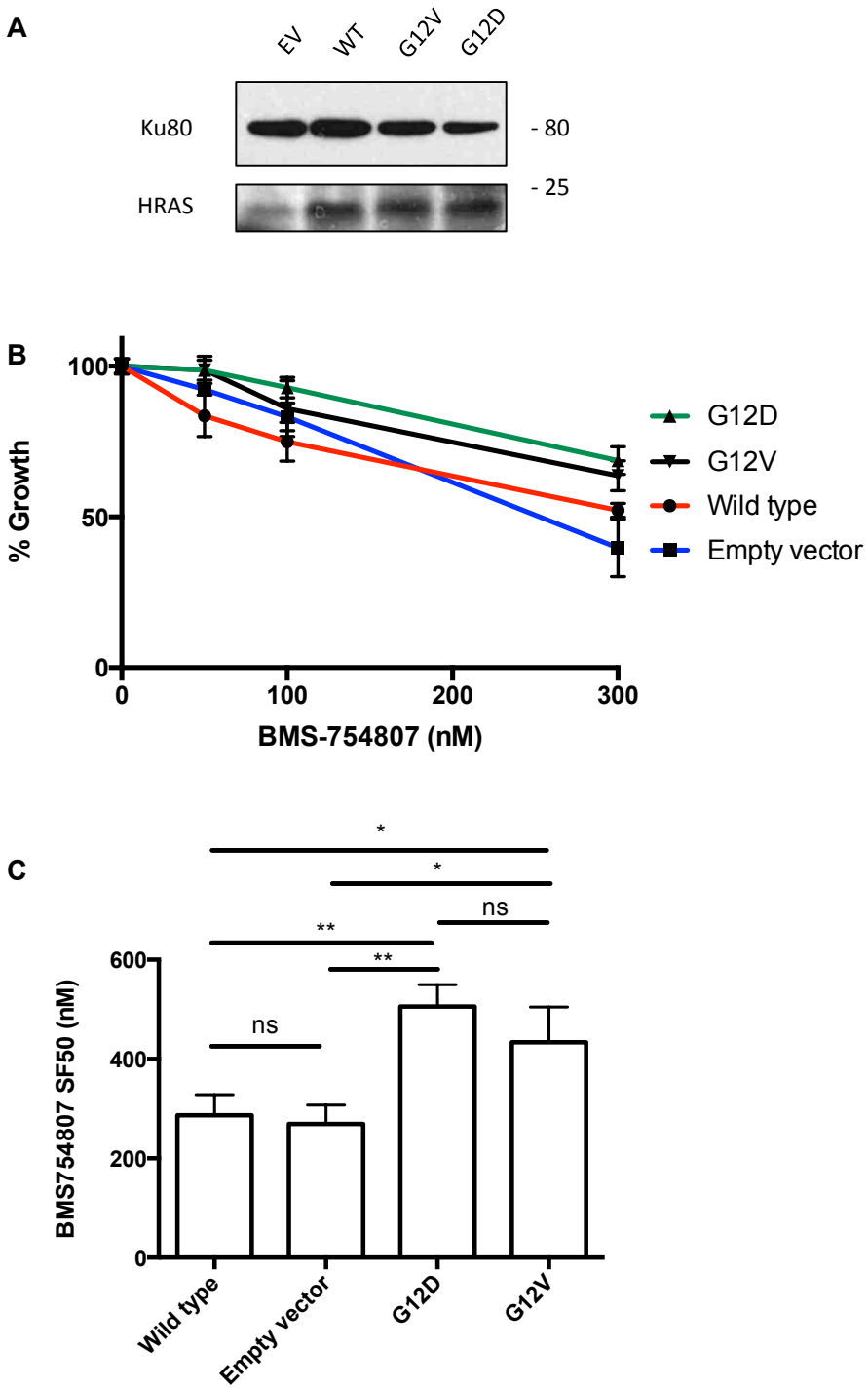


Figure 25: The effect of mutant HRAS expression on response to BMS-754807 in UT-SCC-60A cells.

UT-SCC-60A cells were infected with mutant HRAS (G12D & G12V), wild type HRAS or empty vector. (A) Western blot showing expression of HRAS in infected cells. (B) Clonogenic survival assay showing response of cells to BMS-754807. Points represent mean values ± SEM from 2 independent assays performed in triplicate. (C) SF50 values from clonogenic survival assay; cells transfected with HRAS G12V & G12D were significantly more resistant to BMS-754807 (* $p<0.05$, ** $p<0.01$).

Retroviral infection with HRAS constructs resulted in detectable over-expression of HRAS in both SAS and UT-SCC-60A cell lines (Figure 24A & Figure 25A). In SAS cells, HRAS expression was lower in cells infected with the G12D construct than G12V and wild-type HRAS. Despite this, there was an increase in pERK in both G12D and G12V mutant cell lines compared to wild type or empty vector controls. Although the BMS-754807 SF_{50} of parental SAS cells was 1.59μM (Table 22), the sensitivity of EV and WT HRAS expressing cells to BMS-754807 appeared to be enhanced (SF_{50} = 0.49μM and 0.54μM respectively). This may reflect the toxicity of the infection protocol. SAS cells infected with HRAS G12V were significantly more resistant to IGF-1R inhibition with BMS-754807 in both clonogenic survival assay ($p<0.01$) and CellTiter Glo proliferation assay ($p<0.001$), than those infected with HRAS G12D, wild type or empty vector. In UT-SCC-60A cells infected with empty vector or WT HRAS, the SF_{50} values for BMS-754807 were 260nM and 280nM respectively, which are higher than the value of 40nM obtained in parental cells (Table 22). However, the effect of mutant HRAS expression was similar to SAS: both G12D and G12V HRAS constructs were associated with resistance to BMS-754807 in clonogenic survival assay ($p<0.01$ and $p<0.05$ respectively).

In order to investigate the downstream signalling changes associated with resistance to IGF-1R inhibition in cell lines transfected with mutant HRAS, western blotting was performed on transfected SAS cells treated with BMS-754807 or solvent control (Figure 26). Cells over-expressing wild type and mutant HRAS (G12D and G12V) showed increased ERK phosphorylation compared to empty vector controls. This occurred independent of IGF-1R inhibition, indeed treatment with BMS-754807 appeared to increase ERK phosphorylation compared to untreated cells, in contrast to the inhibition of pERK that had been seen previously (Figure 15). Weak phosphorylation of AKT was detectable in untreated cells, but this was abrogated in cells treated with the IGF-1R inhibitor. These findings suggest that cells transfected

with mutant HRAS (and to a lesser extent wild type HRAS) exhibit increased ERK phosphorylation. It is possible that inhibition of AKT by BMS-754807 may have increased signalling via the MAPK pathway in this context. Despite this effect, BMS-754807 did cause dose-dependent inhibition of growth and survival in SAS cells (Figure 24).

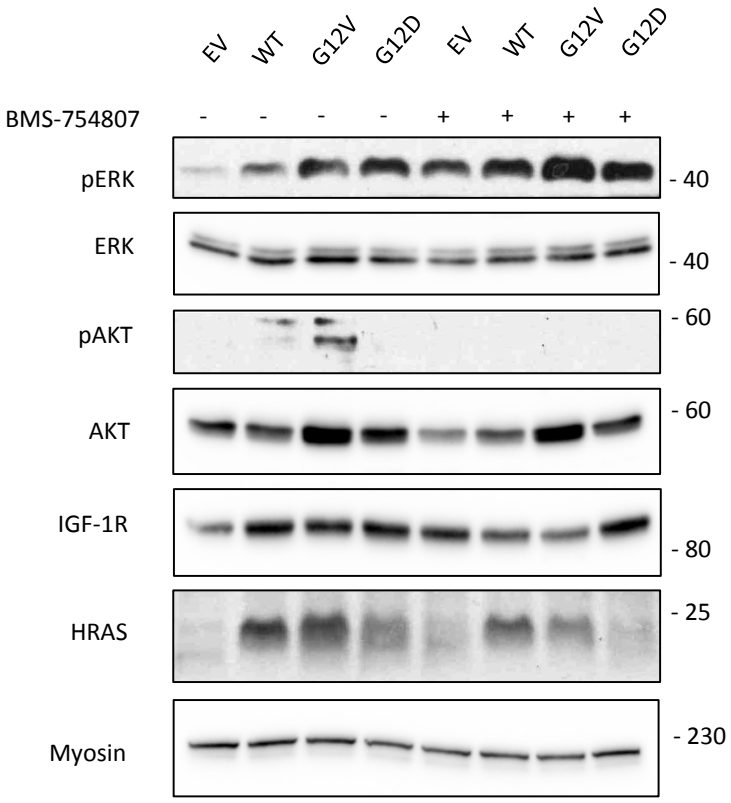


Figure 26: The downstream effects of IGF-1R inhibition in SAS cells over-expressing wild-type or mutant HRAS.

SAS cells were infected with HRAS constructs, and serum starved for 24 hours. Cells were treated with 1μM BMS-754807 or solvent control (0.01% DMSO) for 1 hour (Cells were not stimulated with IGF-1 ligand). Cells were scraped into ice cold PBS, pelleted and lysed for western blotting. Western blot shows signalling changes associated with BMS-754807 treatment in SAS cells (EV: empty vector, WT: wild type HRAS, G12V: HRAS G12V, G12D: HRAS G12D).

These results suggest that activating HRAS mutation is associated with resistance to IGF-1R inhibition as monotherapy in HNSCC cells. Previous results in this chapter have demonstrated that IGF-1R inhibition enhances the radiosensitivity of HNSCC cells, and potentiates the effect of EGFR inhibition (Figure 18 & Figure 23). In order to determine whether HRAS mutation status is predictive of response to IGF-1R inhibition in combination with these established treatments in HNSCC, clonogenic survival assays were performed using EGFR inhibition or irradiation in combination with BMS-754807 in HRAS over-expressing SAS cells.

Cells were infected with retrovirus encoding EV, WT or mutant HRAS and treated with 1μM BMS-754807 for four hours then irradiated at doses of 2-10Gy as previously described (Materials and Methods, Section 2.5.4). Results were obtained from a single assay, and thus must be interpreted with caution, however, they indicate that IGF-1R inhibition with BMS-754807 caused sensitisation to irradiation in wild type and empty vector controls. In cells expressing mutant HRAS (G12V or G12D), no radiosensitisation was seen (Figure 27). This suggests that constitutively active HRAS mutation in HNSCC cells attenuates the ability of IGF-1R inhibition to induce radiosensitisation. Although the mechanism for this has not been fully elucidated, this may result from the increased levels of ERK phosphorylation observed in HRAS mutant cells (Figure 26), which were not supressed by IGF-1R inhibition, and which drive pro-survival pathways (Chitnis et al., 2008). This finding may be of relevance when considering IGF-1R inhibitor trials in HNSCC patients, and suggests that IGF-1R inhibition may act as a more effective radiosensitising agent in patients whose tumours harbour wild-type HRAS than those whose tumours harbour activating HRAS mutations.

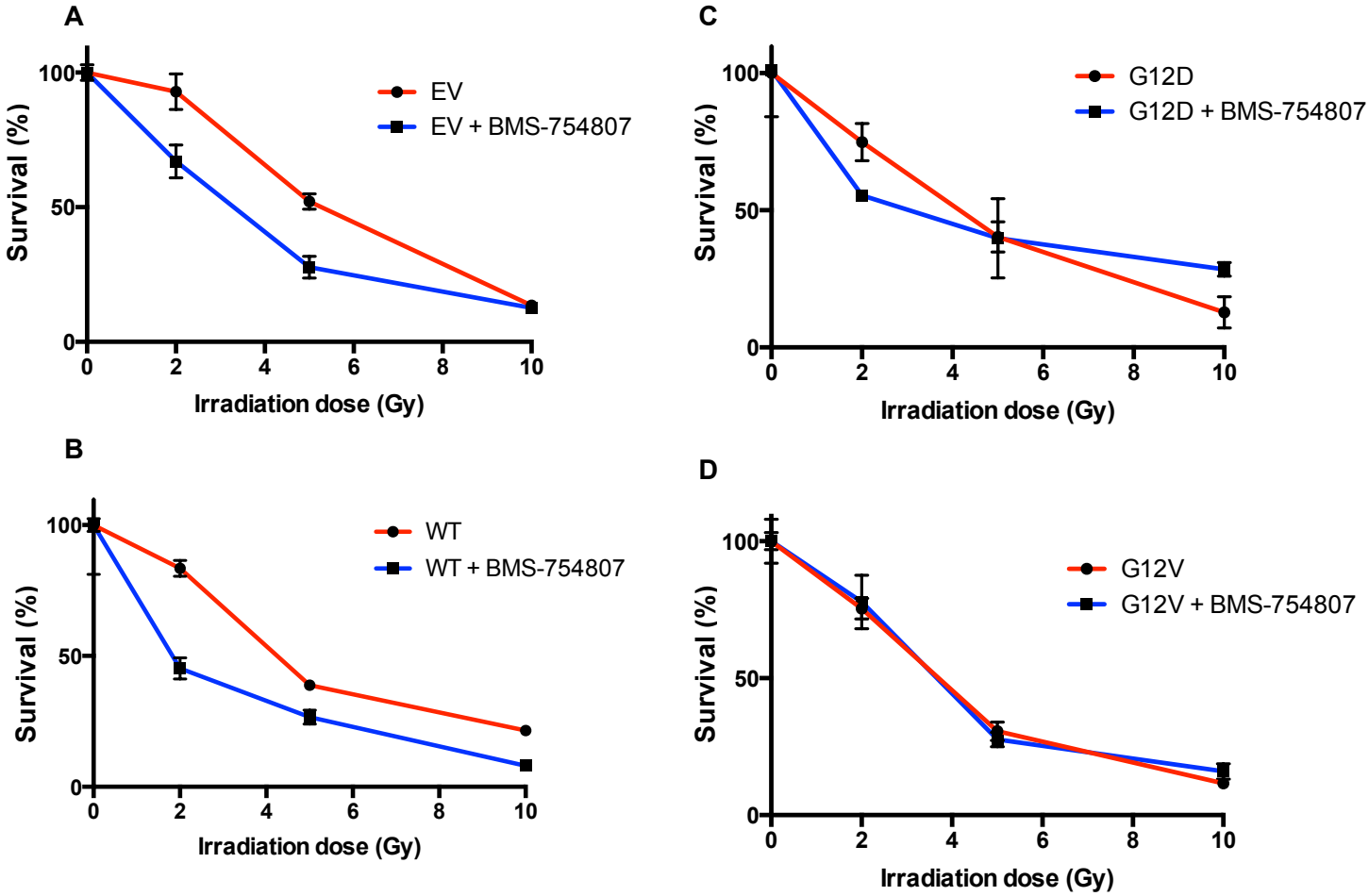


Figure 27: The effect of HRAS mutation status on the ability of IGF-1R inhibition to radiosensitise SAS cells.

SAS cells were infected with empty vector (EV) (A), wild-type HRAS (WT) (B), HRAS G12D (G12D) (C) or HRAS G12V (G12V) (D), and seeded in clonogenic survival assay. After 24 hours cells were treated with 1 μM BMS-754807 or solvent control (0.01% DMSO). Cells transfected with mutant HRAS were not radiosensitised by BMS-754807. Graphs show mean ± SEM cell survival as a percentage of un-irradiated cells and represent data from one assay with triplicate data points.

Co-inhibition of both IGF-1R and EGFR in HNSCC has shown promising results in this and other studies (Barnes et al., 2007, Clayburgh et al., 2013). Moreover, Hah et al. recently demonstrated that activating HRAS mutation is associated with resistance to EGFR inhibition in HNSCC. The final part of this study therefore sought to determine whether HRAS mutation was associated with resistance to co-inhibition of IGF-1R and EGFR in HNSCC cells.

SAS cells expressing EV or WT or mutant HRAS were treated with BMS-754807 and Gefitinib both alone and in combination at SF_{75} concentrations (BMS-754807 SF_{75} = 300nM, Gefitinib SF_{75} = 400nM). The results are shown in Figure 28. These represent triplicate data from a single experiment, and therefore must be interpreted with caution. Although both inhibitors were used at previously determined SF75 concentrations, the use of each inhibitor individually did not reduce cell survival to 75% (BMS-754807 mean survival EV cells = 85.6%, WT cells = 82.6%, Gefitinib mean survival EV cells = 96.2%, WT cells = 81.2%). The effect of co-inhibition of IGF-1R and EGFR was supra-additive, with mean survival in cells co-treated with BMS-754807 and Gefitinib of 59.2% in cells infected with EV and 57.7% in cells over-expressing WT HRAS (Figure 28). Infection of SAS cells with activating HRAS mutant constructs G12D and G12V resulted in relative resistance to combination treatment with BMS-754807 and Gefitinib compared to empty vector and wild-type HRAS controls; Treatment with BMS-754807 and Gefitinib resulted a mean survival fraction of 57.7% in HRAS wild-type cells, 78.2% in HRAS G12D cells ($p<0.001$) and 80.6% in HRAS G12V cells ($p<0.01$).

Although the change in survival upon dual inhibition of IGF-1R and EGFR in HRAS mutant cells was relatively modest, the results suggest that activating HRAS mutations confer resistance to dual IGF-1R/EGFR inhibition, as well as single inhibitor treatment in HNSCC cells.

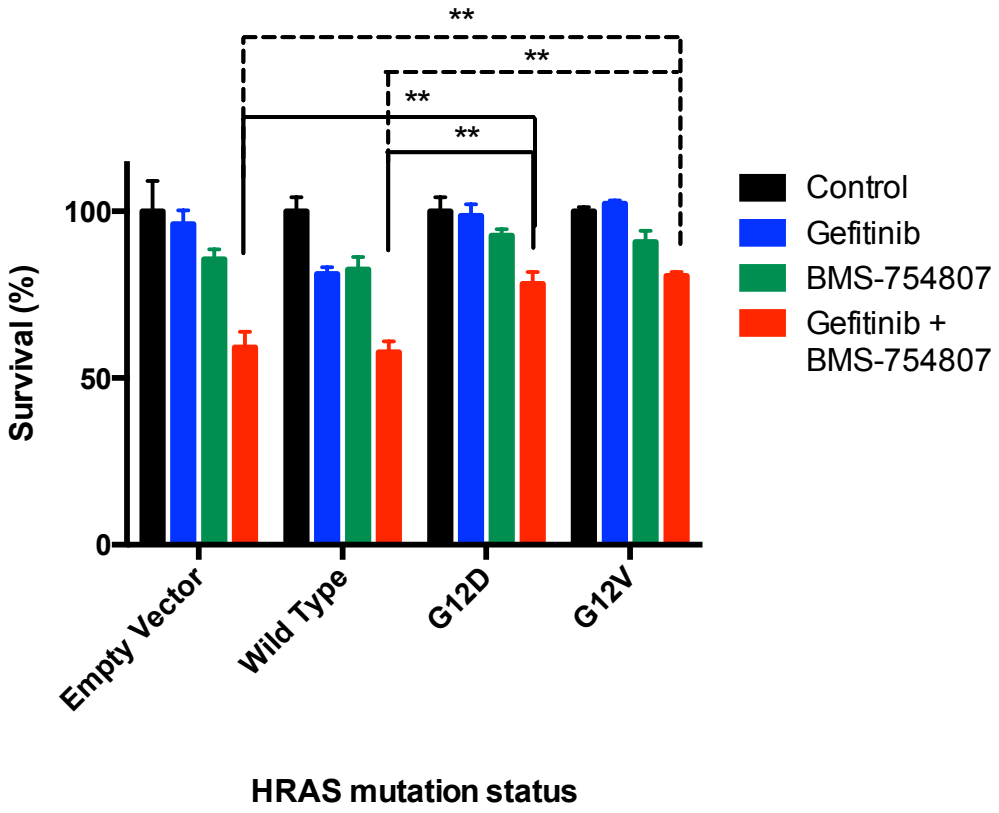


Figure 28: The effect of HRAS mutation status on sensitivity to IGF-1R and EGFR inhibition both alone and in combination in SAS cells.

SAS cells infected with HRAS constructs were seeded for clonogenic survival assay, and after 24 hours were treated with Gefitinib 400nM, BMS-754807 300nM, a combination of both agents or solvent control (0.01% DMSO). Cells were fixed when colonies reached 50 cells. Bars represent mean ± SEM cell survival from one assay with triplicate data points. A t-test was used to determine differences between groups. Cells infected with mutant HRAS constructs were significantly more resistant to combined IGF-1R/EGFR inhibition than EV & WT controls (**p<0.001).

5.11 Discussion

The results from this chapter show that the sensitivity of HNSCC cells to IGF-1R inhibition varies considerably. This variation has been demonstrated in previous studies in both HNSCC and other tumour types, and suggests that predictive biomarkers are urgently required (Huang et al., 2009, Shin et al., 2013).

5.11.1 IGF-1R as a biomarker in HNSCC

Recently, components of the IGF axis have emerged as possible biomarker candidates for predicting sensitivity to IGF-1R inhibition. In colorectal and breast cancer models, IGF-1R, IRS-1 and IRS-2 have been shown to be predictors of biological response to the IGF-1R monoclonal antibody h10H5 (Zha et al., 2009). In sarcoma, which has known sensitivity to IGF-1R inhibition, preclinical data suggest that sensitivity of sarcoma cells to IGF-1R inhibition is associated with high levels of IGF-1R expression (Huang et al., 2009). The utility of these biomarkers, may however, be specific to a particular tumour type or subtype; in HER positive breast cancer, IGF-1R expression is not predictive of response to the IGF-1R tyrosine kinase inhibitor BMS-536924 (Browne et al., 2012). Following correlation analysis of cell line phenotype and protein expression in this study, IGF-1R expression was not associated with sensitivity to IGF-1R inhibition with BMS-754807 (r=0.25, CI-0.7 to 0.88, p=0.63). These data suggest that IGF-1R expression may have limited utility as a predictive biomarker for sensitivity to IGF-1R expression in HNSCC models, however, there were several important limitations to the current analysis. Firstly, the small number of cell lines used means that associations may fail to reach statistical significance. Secondly, IGF-1R levels detected by western blotting are not necessarily indicative of tumoral IGF-1R expression; IGF-1R has been detected by western blotting in tumours considered to be 'IGF-1R null' on immunohistochemistry. This may however reflect the insensitivity of the immunohistochemistry protocols

used in these studies (Schwartz et al., 2013, King et al., 2014). The utility of IGF-1R as a biomarker for IGF-1R inhibition response in a clinical setting is therefore yet to be determined.

The value of IGF-1R as a predictive biomarker may also relate to the subcellular localisation of the receptor, in addition to the level of receptor expression. In a small case-series of 16 patients with soft tissue sarcomas, Asmane et al. showed that clinical response to IGF-1R inhibition was associated with high levels of tumoural nuclear IGF-1R (Asmane et al., 2012b). Previous in-vitro work from our group has demonstrated that IGF-1R undergoes ligand induced translocation from the cell membrane to the nucleus and that nuclear IGF-1R is associated with reduced survival in clear cell renal cancer (Aleksic et al., 2010). Other work has however suggested that it is membrane bound IGF-1R that predicts sensitivity to IGF-1R inhibition. Kim et al showed that IGF-1R requires N-linked glycosylation for transport to the cell membrane, and impaired glycosylation was associated with reduced insertion of IGF-1R into the cell membrane and reduced sensitivity to the IGF-1R monoclonal antibody figitumumab (Kim et al., 2012).

In HNSCC, the significance of the subcellular localisation of IGF-1R remains unclear. Data from this study (Chapter 4) demonstrate that both membrane and cytoplasmic IGF-1R are associated with reduced survival at 5 years, but nuclear IGF-1R was not detectable following immunostaining for IGF-1R in any HNSCC tissue. The relationship between sensitivity to IGF-1R inhibition and membrane/cytoplasmic IGF-1R expression in HNSCC cells was not tested, and represents an avenue for further study.

5.11.2 Dual IGF-1R & EGFR inhibition

Experiments shown in Figure 22A & Figure 23A highlight the supra-additive effect of co-inhibition of IGF-1R and EGFR in HNSCC cells. This in-vitro effect has previously been observed by other authors in HNSCC, and is likely to relate to the significant cross-talk between the IGF-1R and EGFR axes (Barnes et al., 2007, Clayburgh et al., 2013). Evidence for the close association between IGF-1R and EGFR in HNSCC is provided by Barnes et al, who showed that IGF ligand stimulation of HNSCC cells causes IGF-1R/EGFR heterodimerisation and activation of both IGF-1R and EGFR (Barnes et al., 2007). Further support comes from a Phase III trial in which unselected palliative HNSCC patients were treated with figitumumab. Treatment with the IGF-1R monoclonal antibody did not affect progression free survival, but did lead to increased tumoural EGFR phosphorylation (Schmitz et al., 2012). Despite the close association between EGFR and IGF-1R signalling in HNSCC, and some promising results from translational studies, no clinical trials investigating the benefit of dual receptor inhibition in HNSCC patients have been completed. A Phase II trial comparing progression free survival in HNSCC patients treated with Cetuximab and the IGF-1R tyrosine kinase inhibitor OSI906 versus cetuximab and placebo (NCT01427205) was recently terminated prior to enrolment.

Data from this Chapter indicate that IGF-1R inhibition caused variable radiosensitisation of HNSCC cell lines (Figure 18). Despite significant inverse associations between EGFR/pEGFR expression and the ability to radiosensitise with BMS-7548007, knockdown or inhibition of EGFR did not increase the degree of radiosensitisation achieved with IGF-1R inhibition in this study. This suggests that combined IGF-1R:EGFR inhibition may not be an effective approach to enhance the effect of irradiation in HNSCC cells. Indeed while this study was in progress, a report was published investigating the effect of dual IGF-1R/EGFR inhibition on radiosensitivity of HNSCC. Using mouse models, Raju et al compared the effect of

the EGFR antibody cetuximab, the IGF-1R monoclonal antibody IMC-A12 and combination treatment on radiosensitivity of tumour xenografts (Raju et al., 2014). They found that radiosensitivity was enhanced by IMC-A12 or cetuximab but the combination had no additional radiosensitising effect over either agent alone. Although the study used different drugs, these results are similar to those observed in this study.

5.11.3 HRAS as a biomarker of IGF-1R sensitivity

The contrast between the encouraging results of IGF-1R inhibition in preclinical studies and the disappointing results from Phase II/III trials in solid tumours (Langer et al., 2014, Schmitz et al., 2012) suggests that clinical biomarkers indicating sensitivity to IGF-1R inhibition are needed. The results from this study suggest that HRAS mutation status may represent one such biomarker. HRAS mutations have been reported in up to 22% of HNSCC (Anderson et al., 1994), and next generation sequencing provides a reliable and rapid method of genotyping both cell lines and clinical tumour tissue. In this study, HRAS mutation was associated with resistance to the IGF-1R inhibitor BMS-754807 in both of the cell lines tested (SAS and UT-SCC-60A). Introduction of an HRAS mutation also prevented radiosensitisation in response to BMS-754807, and attenuated inhibitory effects of BMS-754807 with Gefitinib.

RAS activation leads to phosphorylation of both PI3K and MAPK (ERK) pathways in a protein kinase cascade that results in cellular proliferation and survival (Chitnis et al., 2008, De Luca et al., 2012, Rodriguez-Viciana et al., 1994). Activation of these cell survival pathways has previously been implicated in resistance to IGF-1R inhibition. This may occur either through molecular cross talk, which provides a 'workaround' for signal inhibition, or genetic alterations, which result in constitutive

activation of downstream effector proteins (Huang et al., 2009). Shin et al determined that *de novo* resistance to the IGF-1R monoclonal antibody cixutumumab in HNSCC cells was associated with phosphorylation of Src and EGFR, with subsequent activation of the PI3K and MAPK pathways (Shin et al., 2013). This again highlights the significance of EGFR/IGF-1R cross-talk in resistance to inhibition of either receptor. In addition to receptor cross-talk, several genetic alterations have also been implicated in resistance to IGF-1R inhibition. Resistance to BMS-754807 has been linked to gene amplification and over expression of PDGFRα, with up-regulation of MAPK signaling in resistant cells (Huang et al., 2010). Similarly, PIK3CA and c-Met mutations are associated with resistance to IGF-1R inhibition with the monoclonal antibody R1507 in small cell lung cancer cell lines, with persistence of AKT phosphorylation despite R1507 treatment (Ferte et al., 2013).

These studies indicate that constitutive activation of signaling proteins downstream from IGF-1R results in resistance to receptor inhibition. Constitutive HRAS activation, and subsequent MAPK/PI3K pathway activity may therefore, lead to resistance to IGF-1R inhibition in much the same way.

Although the results from this and other studies suggest that MAPK/PI3K activity is associated with resistance to IGF-1R inhibition, in this study, neither AKT nor ERK phosphorylation in HNSCC cell lines were significantly associated with resistance to IGF-1R inhibition (pAKT: r=0.78, CI -0.078 to 0.98, p=0.07, pERK: r=0.73, CI -0.21 to 0.97, p=0.1). This may however relate to the small size of the cell line panel tested, as previously described. Support for an association between MAPK signalling and resistance to IGF-1R inhibition in this study comes from the observation that the SAS cell line demonstrated persistent ERK phosphorylation in response to treatment with BMS-754807 and AZ12253801 (Figure 14 & Figure 15), and was highly resistant to IGF-1R inhibition. High levels of ERK phosphorylation in this cell line have previously

been reported, and may relate to over-expression of wild-type KRAS (Saki et al., 2013).

HRAS mutation status has also been reported to have a significant effect on the sensitivity of HNSCC cells to EGFR tyrosine kinase inhibition (Hah et al., 2014). Hah et al. found that the sensitivity of HNSCC cells to erlotinib is governed by the degree of downstream signalling inhibition achieved. They found that constitutively active HRAS resulted in persistence of ERK phosphorylation and resistance to erlotinib, despite abrogation of EGFR phosphorylation. Further evidence for the association between RAS signalling and resistance to EGFR is provided by Saki et al, who showed resistance to cetuximab in HNSCC cell lines overexpressing wild-type KRAS (Saki et al., 2013).

The results from this study suggest that activating HRAS mutation may act as a biomarker for resistance to IGF-1R inhibition in HNSCC cells, both alone and in combination with established treatments. Further evaluation of HRAS as a predictive biomarker *in-vivo* is warranted, and would provide valuable data on the utility of IGF-1R in the clinical setting.

The results presented in Chapters 4 & 5 suggest that IGF-1R is of clinical significance in HNSCC, and is associated with an adverse prognosis in both HPV positive and HPV negative disease. However, the lack of efficacy of IGF-1R as monotherapy in HNSCC (Schmitz et al., 2012) indicates that IGF-1R is likely to be of greatest benefit in combination with other treatments. One such combination may be dual IGF-1R/EGFR inhibition, which caused supra-additive reduction in cell survival in this study. The combination of both agents did not, however, increase the degree of radiosensitisation of HNSCC cell lines over either agent alone. The place of IGF-1R inhibition in the curative treatment of patients with HNSCC therefore remains unclear.

One potential clinical application of combined IGF-1R and EGFR inhibition may be in the palliative setting, in patients who are not fit for chemoradiotherapy. The use of HRAS as a predictive biomarker for sensitivity to treatment may also be useful in this context. In particular, the supra-additive effect of combined treatment may allow dose-reduction, which in turn may alter treatment toxicity. Selection of patients with wild type HRAS would further tailor treatment to those patients likely to derive most benefit. Further work using xenograft models would however be required before such combination therapy entered clinical trials.

6 Conclusion

This study sought to investigate the role of the IGF axis in HNSCC, to determine the prognostic significance of IGF-1R expression in HNSCC, and to identify biomarkers, which may indicate sensitivity to IGF-1R inhibition. The work presented in Chapter 3 sets the context for this research by defining the morbidity and mortality associated with HNSCC. In this chapter, a distinct sub-group of HSNCC patients are examined: those with OPSCC treated with primary surgery ± adjuvant (chemo)radiotherapy. In this group of patients, P16 immunohistochemistry and in-situ hybridization for HPV DNA indicated that 40% of tumours were HPV positive. This figure is slightly lower than other UK data over a similar time period (Evans et al., 2013), but highlights the significant role of HPV in OPSCC. Kaplan-Meier survival analysis showed five and ten-year disease specific survival estimates of 78% and 75% respectively. In patients with HPV positive tumours, both overall and disease specific survival were significantly higher, consistent with published data (Ang et al., 2010).

The chemoradiosensitivity of HPV positive HNSCC has been demonstrated both *in-vitro* and *in-vivo,* and this enhanced treatment response is though to explain the improved survival outcomes in this group of patients (Rieckmann et al., 2013, Ang et al., 2010). The results from this study, add weight to evidence that HPV status is of prognostic significance in patients with OPSCC treated by primary surgery ± adjuvant therapy (Licitra et al., 2006). This is important to consider when deciding on treatment modality in the context of HPV status. This study also highlights that survival rates in HPV negative HNSCC remain poor. The survival rates in this group of patients have changed little over the last 30 years, and novel therapeutic approaches, particularly in HPV negative disease remain a high priority.

Chapter 3 also describes the morbidity associated with the surgical treatment of OPSCC, using validated patient reported outcome measures. These indicate a long-term reduction in quality of life at a mean of 93 months after completion of treatment. Several variables including increasing age, large tumour size, open surgery and free flap reconstruction were associated with adverse outcome in this group of patients. In general, however, questionnaire responses indicated a moderate to good level of function following treatment, as evidenced by the absence of gastrostomy tube dependence at final follow up.

The long-term morbidity of current treatment regimens in HNSCC have stimulated a raft of new clinical trials, investigating treatment de-escalation in patients with favorable disease characteristics (Mirghani et al., 2015). Several of these trials involve the use of targeted agents in place of cisplatin-based chemotherapy, with the aim of reducing short and long-term toxicity. One such example is the De-ESCALaTE HPV trial, which is a randomized, multicenter, open-label Phase III trial (Mehanna, 2015). In this trial, patients with stage III and IV HPV positive OPSCC are randomized to receive either primary chemoradiotherapy or radiotherapy and cetuximab. Primary endpoints include quality of life outcome measures and evaluation of treatment toxicity, as well as survival. The hypothesis is that the use of EGFR inhibition in combination with irradiation in this group of patients may provide reduced toxicity and improved quality of life outcomes, with equivalent survival. The use of other novel, targeted treatment approaches may therefore have a role in reducing the long-term toxicity of current treatment regimens, and warrants further investigation.

Preclinical data from our laboratory indicate that the IGF axis plays a prominent role in cancer (Chitnis et al., 2008, Turney et al., 2012, Aleksic et al., 2010). In the current

study, IGF-1R immunohistochemistry on a TMA containing cores from 346 HNSCCs showed that the receptor is over-expressed in HNSCC compared to normal tissue, and that HPV positive tumours have significantly lower levels of IGF-1R expression compared to HPV negative tumours. The reason for the difference in the expression of IGF-1R in HPV related disease is uncertain, but may relate to differences in tumour suppressor activity. HPV negative HNSCC is characterised by a high rate of *p53* mutation, by contrast however, the rate of *p53* mutation in HPV positive disease is significantly lower (Lawrence, 2015). Wild-type p53 negatively regulates IGF-1R, by suppressing *IGF-1R* promoter activation (Werner et al., 1996), which could explain the lower expression of IGF-1R in HPV positive tumours seen in this study. Although the HPV oncoproteins E6 & E7 are known to inhibit p53 and Rb (Leemans et al., 2011), the effect on *IGF-1R* gene transcription is not fully understood. Further research is required to investigate of the molecular basis of the differences in IGF-1R expression in HPV positive and HPV negative HNSCC.

The relationship between IGF-1R expression and survival is complex, and is influenced by the associations with other major prognostic variables including HPV status and tumour T stage. These interactions have not been fully explored in other studies investigating the effect of IGF-1R expression on survival in HNSCC (Lara et al., 2011, Matsumoto et al., 2014). In the current study, when other prognostic variables were included in multivariate analysis, IGF-1R was not independently associated with survival. The associations with HPV negative status and higher tumour T stage, however, suggest that high levels of IGF-1R expression are associated with an aggressive phenotype in HNSCC. High levels of IGF-1R expression are also associated with adverse outcome and resistance to both ionising radiation and chemotherapy in other cancer types (Lloret et al., 2007, Turner et al., 1997b). The findings of this study therefore raises the question as to whether low levels of IGF-1R may play a role in the relative chemoradiosensitivity of HPV positive

HNSCC. The relationship between IGF-1R expression and survival however, does not necessarily imply that targeting IGF-1R can reverse this phenotype; IGF-1R may be a passenger rather than a driver of resistance in this context.

Although treatment de-escalation is being considered for patients with favourable prognosticators in HNSCC, for patients with HPV negative disease, and indeed for a subset of patients with HPV positive disease (Ang et al., 2010), survival outcomes remain poor, and treatment escalation should be considered. The data presented in Chapter 5 suggest that IGF-1R inhibition reduces HNSCC cell survival *in-vitro* and causes variable sensitisation to ionising radiation as previously reported (Barnes et al., 2007, Riesterer et al., 2011b). In the clinical setting, however, monoclonal antibodies targeting IGF-1R have shown limited single-agent benefit in solid tumours, including HNSCC (Schmitz et al., 2012). Therefore the focus of investigation has recently shifted towards the identification of biomarkers of sensitivity to IGF-1R inhibition (King et al., 2014). The results from this study suggest that activating *HRAS* mutations may represent a biomarker of resistance to IGF-1R inhibition. In SAS cells treated with BMS-754807, infection with mutant *HRAS* G12V increased the BMS-754807 SF_{50} by a factor of 2, and increased the GI_{50} by a factor of 3 compared to WT and EV controls. *HRAS* mutation was also associated with a reduced ability IGF-1R inhibition to sensitise HNSCC cells to irradiation, and with resistance to combined IGF-1R/EGFR inhibition. The finding that HRAS mutation is predictive of resistance to combined IGF-1R and EGFR inhibition supports the work of Hah and colleagues, who demonstrated that HRAS mutation is associated with resistance to the EGFR inhibitor erlotinib in HNSCC cells (Hah et al., 2014). Although resistance mechanisms were not investigated in this study, the persistence of ERK phosphorylation in mutant cell lines treated with BMS-754807 suggests continued activity of pro-survival pathways despite IGF-1R inhibition. These results are consistent with previous

studies, which have demonstrated that the activation of downstream signalling pathways is associated with resistance to IGF-1R inhibition (Huang et al., 2009, Shin et al., 2013). Taken together, these results indicate that the utility of *HRAS* mutation as a biomarker warrants further investigation in the clinical setting.

6.1 The future: Targeted therapy in HNSCC

The results from this study indicate that targeted therapy using tyrosine kinase inhibitors offers promise in the treatment of patients with HNSCC. These agents target specific signalling pathways within HNSCC cells, exploiting the dependence of cancer cells on growth factor signalling. Other strategies for targeting HNSCC cells have also recently been developed. Particular interest has focussed on the role of the host immune system, and ways in which this can be used to target HNSCC cells. Tumour antigens (TAs) refer to a group of antigens and proteins produced by cancer cells. They include onco-foetal proteins, mutated proteins, differentiation antigens and viral proteins, which play an important role in the development and progression of several types of cancer including HNSCC (Hoffmann et al., 2005, Ishii et al., 2015). Following release from tumour cells TAs are taken up by antigen presenting cells (APCs) and bound by major histocompatibility complexes (MHCs). These in turn activate cytotoxic T cells and T helper cells, which release perforin and granzymes, resulting in apoptosis of cancer cells. This process has recently been exploited in the development of cancer vaccines to treat HNSCC. In a phase II trial in patients with HNSCC, Yoshitake and colleagues used a vaccine containing peptides from three TAs to stimulate an immune response against tumour cells (Yoshitake et al., 2015). The TAs IMP3, LY6K and CDCA1 are capable of inducing cytotoxicity in HLA-A24 positive patients (60% of Asian, 20% of Caucasian & 12% African people are HLA-A24 positive). The authors found that HLA-A24 positive vaccinated patients had a

significantly longer median survival time than those vaccinated patients who were HLA-A24 negative (Median survival 4.9 vs. 3.5 months; $p < 0.05$). Vaccinated HLA-A24 positive patients also showed higher levels of TA specific cytotoxic T cell activity (Yoshitake et al., 2015).

The tumour antigens E6 and E7 present in HPV positive HNSCC cells have also been identified as potential targets for cancer immunotherapy. Sewell and colleagues transformed the immunogenic bacterium listeria monocytogenes to express the viral oncoprotein E7. This was administered to C57BL/6 mice with E6/E7 positive tumours and resulted in complete tumour regression in 75% of cases (Sewell et al., 2004). These results however, have not yet been successfully translated to the clinical setting; a recent Phase I trial was closed early due to a serious adverse reaction in a participant (International Standard Randomised Controlled Trial Number 47069182).

Another means of immune modulation in HNSCC may be achieved through targeting PD-1, an immune checkpoint T cell receptor. PD-1 is activated by the ligands PD-L1 and PD-L2, and results in T cell downregulation, which is thought to contribute to the immune evasion demonstrated by several types of cancer including HNSCC (Ferris, 2015). PD-1 is over-expressed in HNSCC, and therefore represents an attractive therapeutic target in this setting (Jie et al., 2015). At the 2015 meeting of the American Society of Clinical Oncologists, early phase clinical data were presented on the efficacy of PD-1 inhibition in 137 patients with HNSCC (Seiwert et al.). Following treatment with Pembrolizumab, tumout regression was seen in 57% of patients. Responses were seen in both HPV positive and HPV negative tumours, and treatment was generally well tolerated by patients, with serious immune-related side effects occurring in less than 10%. These data indicate that immune modulation may provide a novel therapeutic strategy in the management of HNSCC.

6.2 Further research into IGF-1R in HNSCC

The association between HPV positive HNSCC and low levels of IGF-1R expression identified in Chapter 3 suggests that low IGF-1R may play a role in the radiosensitivity of HPV positive disease. This hypothesis could be tested *in-vitro* using HPV positive HNSCC cell lines. Using an isogenic model, upregulation of IGF-1R could be achieved by introduction of an IGF-1R transgene using a plasmid or viral construct. The effect of IGF-1R expression on radiosensitivity in HPV positive cells could then be tested, and a rescue experiment performed using IGF-1R siRNA. Further information on the relationship between IGF-1R and HPV status could be obtained by performing siRNA knock down of the viral oncogenes E6 and E7 in an HPV positive HNSCC cell line and testing the effect on IGF-1R expression and radiosensitivity.

Data from Chapter 3 suggest that high levels of IGF-1R expression are associated with reduced survival in HNSCC, and sub-group analysis indicates that this association persists when HPV positive disease is considered separately. In addition to IGF-1R and HPV, several other molecular markers are known to be of prognostic significance in HNSCC including EGFR and hypoxia inducible factor 1 alpha (HIF-1α) (Winter et al., 2006, Chandarana et al., 2012). It is possible therefore, that IGF-1R may have a role as part of a 'biomarker panel' for determination of high-risk HNSCC patients. Identification of high-risk patients on the basis of tumour markers would allow treatment escalation in this group, which may improve survival outcomes.

The in-vitro data from this work suggest that IGF-1R inhibition may have some clinical utility in the management of patients with HNSCC, particularly in combination with other therapeutic agents. The results from the 2012 GORTEC trial however, indicate that IGF-1R inhibition as monotherapy has limited clinical utility in unselected

patients (Schmitz et al., 2012). Future IGF-1R trials in HNSCC are therefore likely to focus on combination treatment and the use of predictive biomarkers.

The use of IGF-1R inhibition as a radiosensitising agent may be of value in selected patients with HNSCC. The survival benefit of concomitant chemotherapy in addition to radiotherapy in HNSCC has been previously described (Section 1.1.5). A significant proportion of patients though, are not suitable for chemotherapy and in these patients, EGFR inhibition may be considered as an alternative route to radiosensitisation. Resistance to EGFR inhibition is however, emerging as a significant problem in HNSCC (Wang et al., 2014), and in these patients IGF-1R inhibition may allow radiosensitisation.

Another clinical application of IGF-1R inhibition may be in the palliative setting. The results from this study indicate that IGF-1R and EGFR co-inhibition results in a supra-additive reduction in cell survival, which is increased in cells harbouring wild-type *HRAS*. Consideration could therefore be given to performing a randomised-controlled trial in patients with palliative HNSCC, in which they are treated with an IGF-1R inhibitor, EGFR inhibitor or combination therapy, with primary end points being disease response rate and progression free survival. Genetic profiling of tumour specimens would allow stratification of patients by *HRAS* status, which may influence sensitivity to both agents.

To conclude, the work presented in this study highlights the significant morbidity and mortality associated with HNSCC. The association between IGF-1R expression and adverse prognostic indicators suggests that the IGF axis may play a significant role in the biology of HNSCC and therefore represents an attractive therapeutic target. Finally, IGF-1R inhibition has shown considerable promise both alone and in combination with other treatment modalities *in-vitro*, and further testing in the clinical setting is warranted.

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